Intestinal function in cholestasis and essential fatty acid deficiency

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TABLE OF CONTENTS

CHAPTER 1

General introduction

INTRODUCTION

Cholestatic liver diseases comprises a wide range of conditions characterized by defective bile formation and, therefore, impaired bile salt transport from the liver to the intestinal lumen. Physiological consequences include retention of bile salts in the hepatocytes, limited availability of bile salts in the intestinal lumen, hyperbilirubinemia and elevated plasma bile salt levels. These consequences eventually lead to liver injury, lipid malabsorption, jaundice, pruritus and potentially peripheral tissue injury. As a result of malabsorption, cholestatic patients, especially children, can develop serious nutritional defects. For instance, cholestatic disorders are frequently associated with essential fatty acid (EFA) deficiency. The resultant malnutritional state strongly affects prognosis and treatment outcome of cholestatic patients [1]. Identifying specific nutritional defects associated with cholestasis and EFA deficiency and the mechanism(s) underlying these defects will open possibilities towards successful therapies.

In this general introduction, an overview of bile salt metabolism in health and disease (cholestasis) as well as bile salt functions in lipid metabolism will be provided. Processes involved in lipid and carbohydrate absorption from the intestine will be discussed and, finally, the aim of the work described in this thesis will be defined.

BILE SALT SYNTHESIS

Bile acids are present in the form of sodium salts under physiological conditions prevailing in the body (pH 5-7.5) and therefore will be referred to as bile salts. Bile salts are synthesized from cholesterol via the classical (neutral) pathway or the alternative (acidic) pathway. A schematic image of bile salt synthesis is depicted in **Figure 1**. The classical pathway involves modification of the sterol nucleus including saturation of the double bond, epimerization of the 3β-hydroxyl group and hydroxylation at the 7α and 12α-positions, preceding oxidative cleavage of the side chain. Microsomal cholesterol 7α-hydroxylase (CYP7A1) catalyzes the first and rate-controlling step, yielding 7α-hydroxycholesterol which is subsequently converted into 7α-hydroxy-4-cholesten-3-one by micosomal HSD3B7 (3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase). 7α-Hydroxy-4-cholesten-3-one can either enter the pathway towards synthesis of cholic acid (CA) or the pathway towards synthesis of chenodeoxycholic acid (CDCA). Microsomal sterol 12α-hydroxylase (CYP8B1) catalyzes the hydroxylation of 7α-hydroxy-4-cholesten-3-one into 7α,12α-dihydroxy-4-cholesten-3-one, the precursor of CA. The cytosolic enzymes AKR1D1 ($\Delta^{4\text{-}3}$-oxosteroid-5β-reductase) and AKR1C4 (3α-hydroxysteroid dehydrogenase) catalyze reduction of 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one, yielding 5β-cholestan-3α,7α,12α-triol (CA precursor) and 5β-cholestan-3α,7α-diol (CDCA precursor), respectively. Mitochondrial sterol 27-hydroxylase (CYP27A1) oxidizes the side chains of both precursors, yielding 3α,7α,12α-trihydroxy-5β-cholestanoic acid and 3α,7α-dihydroxy-5β-cholestanoic acid. These precursors are ligated to coenzyme A by bile acid CoA synthetase (BACS) activity and are subsequently transported into the peroxisomes for side chain cleavage. Side chain cleavage eventually leads to the formation of CA (3α.7α,12α) and CDCA (3α,7α). In the alternative pathway, cholesterol is hydroxylated into oxidized intermediates (oxysterols) through the actions of CYP27A1 and oxysterol 7α-hydroxylase (CYP7B1). While the classical pathway is exclusive to the liver, the alternative pathway also occurs in peripheral tissues [2-4]. In rodents, the majority of CDCA is converted to the more hydrophilic bile salt α-

muricholic acid (α-MA) and subsequently to β-muricholic acid (β-MA) in the liver. In rodents, CA and β-MA are the main bile salts present in bile [5,6].

Nearly all bile salts undergo conjugation with glycine or taurine in liver peroxisomes, catalyzed by bile acid-CoA:amino acid *N*-acetyltransferase (BAAT) In humans, the majority of bile salts are conjugated with glycine, while rodent bile salts mainly consist of taurine-conjugates [2-4,7].

A small fraction of the (conjugated) primary bile salts enter the colon, where they can be deconjugated and converted to hydrophobic secondary bile salts by colonic bacteria, or are excreted into the feces. After deconjugation, the primary bile salt CA can be 7α-dehydroxylated into deoxycholic acid (DCA) and CDCA can be 7α-dehydroxylated into lithocholic acid (LCA) [8]. In addition, CDCA can be converted to the more hydrophilic bile salt ursodeoxycholic acid (UDCA) by colonic bacterial oxidation of the 7α-hydroxyl group and stereospecific reduction of the 7-keto group, generating the corresponding 7β-hydroxyl group [9]. In mice and rats, β-MA is converted to hyodeoxycholic acid (HDCA) through 7β-hydroxylation and 6β-hydroxy epimerization and or to ω-muricholic acid (ω-MA) through epimerization of the 6-hydroxy group [5,6,10].

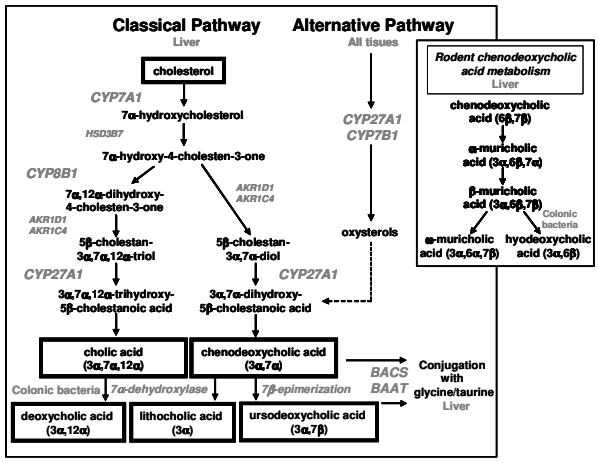

Figure 1 Classical and alternative pathway of bile salt synthesis and rodent chenodeoxycholic acid metabolism.

ENTEROHEPATIC CIRCULATION

Under non-cholestatic conditions, conjugated primary and secondary bile salts are secreted into the bile. Bile functions as a route for the excretion of endogenous and exogenous compounds such as bile salts, bilirubin, phospholipids, cholesterol, drugs and toxins. In humans and mice, bile salts are stored and concentrated in the gallbladder during the interdigestive period and are excreted postprandially to aid in the absorption of lipids, cholesterol and fat-soluble vitamins. The turnover of the bile salt pool is ~5%, that is ~5% is lost in the feces per cycle, and ~95% is (passively and actively) reabsorbed by the intestine. The majority of bile salts is absorbed via active transport in the terminal ileum and transported back to the liver via the portal circulation [11-13].

Hepatic bile formation is driven by the osmotic gradient generated by secretion of relatively non-permeant solutes into the canalicular lumen. Thus, accumulation of osmotically active molecules promotes the movement of water and electrolytes across the canalicular

membrane. Bile salts account at least for 40-50% of bile flow, while the remainder depends on electrolytes [14,15], bicarbonate and mostly gluthathione and its conjugates [16-18]. Most of the gluthathione (70-80%) in bile is in the reduced form (GSH) [15]. **Figure 2** represents a schematic image of the enterohepatic circulation and the bile salt transporters involved.

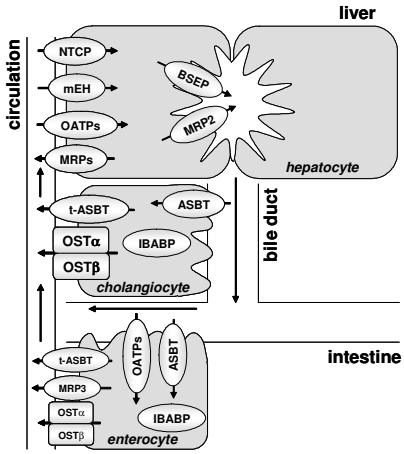

Figure 2 Schematic image of the enterohepatic circulation of bile salts with the bile salt transporters involved.

Bile salt transport across the canalicular membrane into the bile canaliculi occurs against a 100-1000 fold concentration gradient [19]. Canalicular secretion of bile salts involves the actions of transporters whose function depends on ATP hydrolysis. The major transporters involved are Bile Salt Export Pump (BSEP/ABCB11), responsible for transport of monovalent bile salts, and the Multidrug Resistance-associated Protein 2 (MRP2/ABCC2), the main transporter of divalent bile salts [12,13,19-23]. BSEP is essential for canalicular bile secretion, evidenced by knockout mice exhibiting impaired bile secretion and intrahepatic cholestasis. Wang *et al.* demonstrated that secretion of CA (monovalent) was greatly reduced (6% of wild-type), while total bile salt output was about 30% of wildtype mice in mice lacking BSEP [24]. BSEP is exclusively expressed in the liver [25]. BSEP has high affinity for TCDCA > TCA > TUDCA > GCA [26,27]. MRP2 is localized to the canalicular membrane of hepatocytes, the apical membrane of renal proximal tubule epithelial cells, and the apical membrane of duodenal en jejunal enterocytes [28]. Besides divalent bile salts like sulphated TLCA and GLCA, MRP2 mediates the export of bilirubin conjugates, gluthathione, glucuronide and sulphate conjugates, and some unconjugated drugs [12,28-30]. MRP2 does not have the capacity to transport monovalent bile salts [12,31]. MRP2-deficient rats (GY/TR$^-$ and EHBR rats) are characterized by permanent hyperbilirubinemia and strong induction of MRP3 [12,32-34].

Bile salts are actively absorbed by cholangiocytes lining the bile ducts to recycle back to the hepatocytes for their re-secretion (cholehepatic shunt pathway) [12,13,35]. Apical Sodium-dependent Bile Salt Transporter (ASBT) mediates active transport of bile salts from the bile ducts across the apical membrane [36,37]. Ileal Bile Acid Binding Protein (IBABP) is also expressed in cholangiocytes, though its exact function is still unclear [36]. It is suggested that IBABP modulates transcellular transport and prevents intracellular bile salt toxicity by binding bile salts, however, evidence is still lacking [35]. Bile salt efflux via the basolateral

membrane is mediated by t-ASBT, an alternatively spliced form of ASBT [38], Multidrug resistance protein 3 (MDR3/ABCB4) is also expressed at the basolateral membrane of cholangiocytes [39]. However, there is no direct evidence that MDR3 is involved in cholangiocyte bile salt efflux. Recently, the heterodimeric Organic Solute Transporter α/β (OSTα/β) has been indentified in cholangiocytes. OSTα/β can transport bile salts via facilitated diffusion [40,41].

Bile salts that have not been transported back to the liver via the cholehepatic shunt pathway enter the intestinal lumen and are passively and actively absorbed by the enterocytes. Apical uptake of bile salts in the enterocytes occurs via passive diffusion of unconjugated bile salts in the small intestine and the colon (after deconjugation by intestinal microflora), which accounts for a small fraction of intestinal bile salt conservation [42,43]. ASBT is also expressed in the terminal ileum and is responsible for absorption of the majority of luminal bile salts [44-46]. Human ASBT efficiently transports conjugated and unconjugated bile salts with a preference for the taurine and glycine conjugates [47,48]. ASBT mediated transport is electrogenic with 2:1 Na^+/bile salt coupling stoichiometry [49]. ASBT exhibits a higher affinity for dihydroxy bile salts (CDCA and DCA) compared to trihydroxy bile salts such as CA, TCA and GCA [47]. The phenotype of ASBT knockout mice involves intestinal bile salt malabsorption and interruption of the enterohepatic circulation, emphasizing the importance of ASBT [50]. A Na^+-independent bile salt transporter (Organic anion transporting polypeptide; Oatp1a5) was found to be expressed in rat jejunal enterocytes [12]. OATP1A2 has been suggested as the human Oatp1a5 ortholog [51,52]. OATPs transport anions via a Na^+-independent mechanism. Jejunal uptake of bile salts occurs via in-to-out HCO_3^- gradient; however the role of OATPs herein remains unclear [53]. Intracellular transport of bile salts in enterocytes is thought to occur via IBABP. Intestinal IBABP expression is restricted to the terminal ileum [12,54]. No data is yet available about IBABP knockout models, but the observation that downregulation of IBABP expression resulted in increased fecal bile salt loss is suggestive of a function in ileal bile salt absorption [55]. However, Kok *et al.* demonstrated that intestinal bile salt reabsorption was markedly increased in *Fxr*$^{-/-}$ mice despite complete absence of Ibabp, suggestive of a function as a negative regulator of bile salt reabsorption rather than a positive [56]. Concluding, IBABP function in bile salt reabsorption remains unclear. t-ASBT MRP3 and OSTα/β have been proposed as candidates for basolateral bile salt efflux transporters in enterocytes; their specific roles, however, have remained undefined so far [12].

To complete their enterohepatic circulation, absorbed bile salts are transported back to the liver via the portal blood. The basolateral membrane of the hepatocytes is in direct contact with the space of Disse that receives its content from the portal blood through large pores of the sinusoidal endothelium. The majority of bile salts reaches the space of Disse bound to albumin and needs to be dissociated for translocation across the membrane [57]. Na^+-dependent Taurocholate Co-transporting Polypeptide (NTCP) is the main transporter mediating bile salt uptake. NTCP has been shown to mediate the transport of both conjugated and unconjugated bile salts in a Na^+-dependent manner with a stoichiometry of 2:1 [58]. Several studies have also suggested a role for microsomal epoxide hydrolase (mEH) in Na^+-dependent bile salt uptake [12]. However, since mice lacking mEH expression have no apparent abnormalities in bile salt homeostasis, the contribution of mEH to hepatocyte bile salt uptake has become debated [59]. Though, hypercholanemia was associated with a 85% decrease of mEH protein due to a point mutation in a patient with no alteration in NTCP expression [60]. Zhu *et al.* proposed that mEH is more efficient for transporting bile salts

conjugated with glycine compared to NTCP in humans [60]. Since the majority of bile salts in humans are conjugated with glycine, in contrast to rats and mice, it is possible that mEH is responsible for hepatic uptake of a higher percentage of bile salts compared to NTCP [60]. Most unconjugated bile salts are transported in a Na^+-independent manner via passive diffusion or carrier-mediated transport [57,61]. Several members of the OATP family have been implicated in basolateral bile salt influx; OATP1A2, OATP1B1 and OATP1B3 [12,57,62]. OATPs mediate the exchange of extracellular HCO_3^- or gluthathione, indicating that they are also involved in GSH efflux [63].

Under physiological conditions, bile salt transport from the hepatocyte across the basolateral membrane into the portal blood is negligible [51]. Transport is mediated by members of the MRP family. MRP1 (ABCC1), 3 (ABCC3) and 4 (ABCC4) are localized to the basolateral membrane and can transport bile salts and other compounds in a ATP-dependent fashion [12,64,65]. Zelcer *et al.* demonstrated that bile salt homeostasis was unaltered in mice lacking Mrp3, indicating that Mrp3 is not solely responsible for bile salt efflux or may not be involved [66].

Hepatic transcellular transport of bile salts can occur via intracellular traficking and vesicle-mediated transport. Under physiological conditions, the majority of bile salts is transported based on the basolateral to canalicular concentration gradient, possibly be mediated by intracellular bile salt-binding proteins. The involvement of vesicle-mediated transport was concluded from several observations that indicated the partitioning of hydrophobic bile salts into membraneous intracellular organs such as endoplasmic reticulum and Golgi apparatus [42,51].

PHYSIOLOGICAL FUNCTIONS OF BILE SALTS

Physiological functions of bile salts include cholesterol elimination, stimulation of bile flow, stimulation of biliary phospholipid secretion, regulation of bile salt and cholesterol biosynthesis and enhancement of lipid absorption [67]. Apart from their role in dietary lipid absorption and cholesterol homeostasis, bile salts also function as signaling molecules. Through activation of diverse signaling pathways, bile salts can regulate their own enterohepatic circulation, and metabolism of triglycerides, cholesterol, energy and glucose [68]. Bile salts can activate nuclear hormone receptors such as the farnesoid X receptor (FXR) [69-71], involved in the regulation of bile salt metabolism, lipid metabolism and glucose metabolism. The role of FXR in bile salt homeostasis and enterohepatic circulation will be discussed below in combination with cholestasis.

Bile salts have also been shown to affect the microflora and the integrity of the small intestine. Obstruction of bile flow in humans and rodents causes proliferation of intestinal bacteria and mucosal injury, which can lead to bacterial translocation across the mucosal barrier and systemic infection [72]. Bacterial overgrowth and translocation caused by biliary obstruction in rats was inhibited by oral administration of bile salts [73,74]. In addition, oral administration of bile salts blocked endotoxemia in patients with obstructive jaundice [75-77]. Recently, Inagaki *et al.* demonstrated that FXR induces genes involved in enteroprotection, such as iNOS, IL18 and angiogenin, and thereby inhibits bacterial growth and ileal mucosal injury in cholestatic mice. Moreover, mice lacking FXR had increased ileal levels of bacteria and a compromised epithelial barrier [78].

In addition to FXR, bile salts have also been shown to activate other nuclear receptors, the pregnane X receptor, the vitamin D receptor and the constitutive androstane receptor,

leading to a reduction of bile salt toxicity [79-82]. Bile salts can also activate MAPK pathways, usually by activating cellular membrane receptors, and thereby affect proliferation and apoptosis [83-88]. Recently, bile salts were also found to be ligands for a G protein-coupled membrane receptor (TGR5/GPBAR1) [89,90]. Watanabe *et al.* provided data to suggest that bile salts regulate energy metabolism via binding to GPBAR1 [91,92].

CHOLESTASIS

Cholestatic liver disease has been associated with numerous nutritional deficiencies, including EFA deficiency, and subsequent failure to thrive (reviewed in chapter 2) [1]. Despite numerous treatment options to alleviate the clinical manifestations of cholestasis, cholestatic liver disease can necessitate a liver transplantation [93,94]. In children, cholestasis-induced malnutrition or failure-to-thrive is associated with a worse outcome of their disease [95-99]. Cholestatic disorders can be divided in hereditary (genetic) disorders and acquired disorders. The majority of hereditary cholestatic disorders are characterized by defects in transporters involved in bile formation [100-102].

Etiology of cholestatic disorders

Progressive Familial Intrahepatic Cholestasis (PFIC) comprises a group of autosomal recessively inherited disorders associated with transporter defects. Mutations in the *FIC-1 (ATP8B1)* gene, encoding FIC-1, can cause PFIC type 1 (PFIC-1) or Benign Recurrent Intrahepatic Cholestasis (BRIC) [100,102,103]. PFIC-1 is most commonly diagnosed in newborns and often begins with cholestatic episodes progressing to permanent cholestasis with fibrosis, cirrhosis and liver failure in the first two decades of life. PFIC-1 is also associated with jaundice, pruritus, diarrhea and a failure to thrive. Plasma levels of bile salts and cholesterol are elevated [104]. BRIC is characterized by recurrent spells of cholestasis associated with pruritus, jaundice, fatigue, loss of appetite, anorexia and elevated plasma bile salt levels [103,104]. The age of onset, severity and number of these episodes vary greatly [105]. FIC-1 profoundly acts as an aminophospholipid translocase, 'flipping' specific phospholipids (phosphatidylserine and phosphatidylethanolamine) from the outer leaflet to the inner leaflet of the membrane [106,107]. FIC-1 and other homologues have been detected all over the body, including the bile canalicular membrane of hepatocytes and, with higher abundance, in the intestine and cholangiocytes [106,108,109]. Studies with FIC-1 knockout mice revealed disturbed bile salt homeostasis, but only mildly impaired bile secretion, despite elevated plasma bile salt levels [110,111]. The former findings are suggestive of an important role for the intestine in PFIC-1. This was confirmed by the observation that biliary diversion is a successful treatment for PFIC-1 patients, alleviating intrahepatic and extrahepatic symptoms [112,113]. Chen *et al.* demonstrated that *FIC-1* mRNA was absent in the ileum of PFIC-1 patients, coinciding with strongly reduced *FXR* expression and fourfold higher *ASBT* expression. They also showed that BSEP promoter activity was diminished while ASBT promoter activity was greatly enhanced in *FIC-1* antisense treated Caco-2 cells, presumably via the loss of FXR [114]. Therefore, it is likely that bile salt uptake from the ileum is enhanced and bile salt secretion from the liver is diminished in PFIC-1 patients, explaining the manifestation of cholestasis. Studies with Fic1 (Atp8b1) knockout mice, however, did not reveal enhanced bile salt absorption or increased Asbt expression, suggesting that the accumulation of bile salts in the plasma is not caused by increased intestinal bile salt absorption [110]. Moreover, Paulusma *et al.* showed that Atp8b1 deficiency resulted in a loss

of canalicular phospholipid asymmetry that in turn renders the canalicular membrane less resistant toward hydrophobic bile salts, which may impair bile salt transport and cause cholestasis [115].

PFIC-2 is caused by mutations in the *BSEP (ABCB11)*, encoding BSEP, the canalicular bile salt efflux pump [116]. Jansen *et al.* demonstrated that BSEP was absent from the canalicular membrane in PFIC-2 patients [117]. The clinical symptoms of PFIC-2 overlap with PFIC-1, but with fewer extrahepatic abnormalities and generally with a less episodic character, though often with more severe symptoms [102-104]. Bsep knockout mice show a clear decrease in bile salt secretion [24,103], explaining the manifestation of cholestasis.

PFIC-3 is associated with mutations in the *MDR3 (ABCB4)* gene, encoding MDR3 [102-104]. MDR3 is a phospholipid floppase at the canalicular membrane, facilitating the secretion of phosphatidylcholine in bile [118]. The clinical symptoms of PFIC-3 are different from PFIC-1 and PFIC-2 as in that symptoms usually present somewhat later in life and also liver failure occurs at a later age. The histological picture shows strong bile duct proliferation and cirrhosis. PFIC-3 is also associated with jaundice, pruritus and elevated plasma bile salt levels [102-104]. A mouse model in which the mouse homolog of the human MDR3 gene, Mdr2, was disrupted revealed absence of biliary phospholipids despite normal bile salt secretion, progressive liver disease and histology similar to that in PFIC-3 patients [119]. Biliary phospholipids form mixed micelles with bile salts and prevent crystallization of cholesterol. In the absence of phospholipid secretion, the detergent bile salts tend to solubilize lipids from the biliary epithelium and cholangiocytes, leading to bile duct damage and secondary biliary fibrosis and cirrhosis [104].

Inborn errors of bile salt biosynthesis can also lead to cholestasis. Seven defects have been characterized so far, including mutations in the genes encoding for HSD3B7 [120-122], AKR1D1 [123,124], CYP7A1 [125], CYP27A1 [126], CYP7B1 [127], BAAT [128] and 2-Methylacyl-CoA racemase [129]. The phenotype of these defects is highly variable and they do not all lead to cholestasis. Liver disease tends to be more severe in defects that involve modifications of the cholesterol side chain than defects responsible for catalyzing reactions in the steroid nucleus (except for CYP7A1 deficiency) [130].

Finally, the autosomal dominant hereditary Alagille syndrome is the most common form of hereditary cholestatic liver disease in children. Alagille syndrome is associated with mutations in the human JAGGED gene, leading to hypoplasia of the intrahepatic bile ducts among various other defects [131,132]. Clinical manifestations include chronic cholestasis, characteristic facial features, pulmonary artery hypoplasia and congenital heart disease. Severely affected children account for about 20% of the patients and may require liver transplantation [133]. Jagged 1 encodes a ligand for Notch 1, which seems essential for remodeling of embryonic vasculature [134,135].

Acquired forms of cholestasis can be caused by changes in transporter expression or function and, more commonly, by obstructions or destruction of the bile ducts. Changes in transporter or function are usually due to cholestatic agents such as drugs, hormones or pro-inflammatory cytokines. Gallstones and tumors can cause obstruction of extrahepatic bile ducts, while vanishing bile duct syndromes such as biliary atresia, primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are associated with the destruction of bile ducts.

Biliary atresia is characterized by the immune-mediated destruction of the extrahepatic and intrahepatic bile ducts and development of biliary cirrhosis. Biliary atresia is the leading cause of liver transplantation in children. The disorder occurs in 1 in 16,000-18,000 live

births and is slightly more common in females (1.2:1). Biliary atresia can be distinguished in perinatal (acquired) and the embryonic (fetal) types. The majority (80%) of biliary atresia cases are of the perinatal type. These otherwise normal infants are presumably born with a patent biliary system which undergoes progressive inflammation initiated by a perinatal insult. The embryonic form of biliary atresia is associated with other congenital anomalies, most commonly involving abdominal situs inversus (major visceral organs are inversed or mirrored from their normal positions) [136]. The clinical symptoms of both types are similar, though the etiologies may differ [131,137]. Several potential mechanisms involved in the pathogenesis of both forms have been suggested, including defects in morphogenesis, defects in prenatal circulation (embryonic), immunologic dysregulation, viral infection and toxin exposure (perinatal) [137-140].

PBC is an autoimmune disease marked by slow but progressive disappearance of the intrahepatic bile ducts. Both autoreactive T cells and natural killer cells have been associated with the pathogenesis of PBC [141,142]. Also PSC is an inflammatory autoimmune disease leading to the progressive destruction of the biliary tree. Though several mechanisms contributing to PSC have been postulated, including portal bacteremia, viral infections, toxins and ischemic injury, the etiology of PSC remains unknown [131].

Treatment of cholestatic disorders

Current surgical treatment options include biliary drainage for obstructive cholestasis and Kasai-portoenterostomy for biliary atresia. Despite good results, however, the need for liver transplantation remains for many cases.

A convential treatment for cholestasis-induced pruritis is the anion-exchange resin cholestyramine. Cholestyramine is a hydrophilic, water-insoluble, non-absorbable agent that binds bile salts, preventing their absorption from the terminal ileum. Recently, a new bile salt-binding polymer, colesevalem hydrochloride has been reported to be more effective than cholestyramine [143]. Alternative anti-pruritus treatments include rifampicine and naltrexon.

UDCA, at present, is the only approved drug for cholestatic disorders [94], and has been proven beneficial for PBC [144-149], PSC [150-152], PFIC [153] and some forms of drug-induced cholestasis [154]. The beneficial effects of UDCA have been attributed to three major actions: protection of cholangiocytes against cytotoxicity of hydrophobic bile salts, stimulation of hepatobiliary and renal secretory routes, and protection of hepatocytes against bile salt-induced apoptosis.

Hydrophobic bile salts are known to damage cell membranes at high micromolar to millimolar concentrations *in vitro*. UDCA conjugates counteract these effects, by modulating the structure and composition of phospholipid-rich micelles in bile [155,156]. Moreover, enrichment with UDCA renders bile more hydrophilic and less cytotoxic in humans, decreasing the degree of cholangiocellular injury [157]. Ca^{2+} and PKC-dependent mechanisms have been shown to contribute to anticholestatic actions of UDCA conjugates in hepatocytes [158-160]. In cholangiocytes, UDCA might work in a similar fashion.

The disturbance common to all forms of cholestasis is impaired bile production. This results in a retention of bile salts and other potentially toxic biliary constituents in the liver, which can leads to or aggravate liver cell injury with further implication for bile formation and hepatocellular apoptosis [161]. UDCA has been shown to stimulate biliary secretion of bile salts and other organic anions and prevent cholestasis induced by hydrophobic bile salts in the rat liver [160,162].

Apoptosis is the major form of hepatocyte cell death in cholestatic liver diseases such as PBC, and has been attributed to the actions of accumulating hydrophobic bile salts in liver cells [160,162,163]. *In vitro* and *in vivo* studies in rats revealed that UDCA inhibits apoptosis via reduction of mitochondrial membrane permeability transition and of mtochondrial cytochrome c release [164,165]. In addition, UDCA induced survival signaling in hepatocytes via activation of the epidermal growth factor receptor (EGFR) and mitogen activated protein kinase (MAPK) [166].

LIPID ABSORPTION AND METABOLISM

Cholestasis is frequently accompanied by lipid malabsorption and essential fatty acid (EFA) deficiency. Lipids are a large and diverse group of naturally occuring organic compounds that are related by their solubility in nonpolar organic solvents and general insolubility in water. Lipids serve many physiological functions, including energy storage, structural components of cell membranes and signaling molecules. Fahy *et al.* developed a well-defined classification system for lipids. Lipids were divided into eight categories; fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides. The lipids that are discussed here are triglycerides (glycerolipids), phospholipids (glycerophospholipids), fatty acids (fatty acyls) and cholesterol (sterol lipids). Bile salts are also included in the sterol lipid category [167]. Lipid absorption and metabolism can divided in several steps; lipolysis, solubilization, mucosal uptake, re-esterification and chylomicron formation, and lipoprotein metabolism. **Figure 3** shows the chemical structures of triglycerides, phospholipids, cholesterol and bile salts and a schematic overview of lipid and cholesterol absorption and metabolism is given in **Figure 4**.

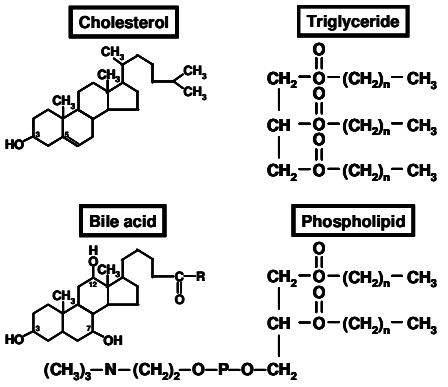

Figure 3 Chemical structures of cholesterol, a bile acid (cholic acid), a triglyceride and a phospholipid (phosphatidylcholine).

Lipolysis

Triglycerides (triacylglycerols) are the major lipid contents in the human diet and constitute about 40% of the energy intake in Western diets. Other dietary lipids include phospholipids, glycolipids, sterols and vitamins A, D, E and K. Prior to lipolysis, the chewing process disperses the lipids, whereby the surface area is increased and a food bolus is formed. The food bolus is transferred to the stomach where partial enzymatic hydrolysis of triglycerides

into diacylglycerol and free fatty acids takes place [168,169]. Hydrolysis is performed by gastric lipase or lingual lipase. Lingual lipase is released from von Ebner's glands, the lingual serous glands on the tongue, and is transferred with the food bolus from the mouth into the stomach where its activity is exerted [170,171]. Gastric lipase is released from the gastric mucosa [172]. The relative contribution of these lipases to hydrolysis depend on the species considered. Rodents have high activity of lingual lipase and low activity of gastric lipase, while primates and humans have high activity of gastric lipase [173]. About 10-30% of dietary triglycerides are hydrolyzed in the stomach in humans and rats [174,175]. The predominant fraction of dietary triglycerides is hydrolyzed by pancreatic lipase. The entry of triglyceride degradation products in the duodenum causes gallbladder emptying and cholecystokinin release with subsequent pancreatic lipase secretion [176-178]. Pancreatic lipase binds to the surface of the food bolus with co-lipase as a mediator to overcome the expulsion of the lipase into the water-phase caused by bile salts and digests the triglycerides. This lipolytic process results in the formation of monoacylglycerol and free fatty acids [179]. Phospholipids in the duodenum are derived from diet, bile and intestinal epithelial sloughing. Pancreatic phospholipase A_2 catalyzes the hydrolysis of a variety of phospholipids in the duodenum, yielding lysophospholipids and free fatty acids [168]. Cholesterol in the intestinal lumen can be derived from diet, from bile and from epithelial sloughing. In response to a meal, carboxyl ester lipase is secreted from the pancreas and catalyzes de-esterification of cholesteryl esters. The pool of unesterfied cholesterol (mainly derived from bile) in the intestinal lumen is relatively much larger than the esterified dietary pool of cholesterol [180]. Accordingly, targeted disruption of the gene encoding for carboxyl ester lipase only has a slight inhibitory effect on intestinal cholesterol absorption in mice [181,182].

Solubilization

The products of pancreatic lipolysis of dietary lipids (fatty acids, monoacylglycerol, lysophospholipids, unesterified cholesterol) are more polar than their parent lipids, but still have limited solubility in the aquous environment of the intestinal lumen. Bile provides the ideal detergent for the solubilization of these lipolytic products. The amphipathic nature of bile salts allows them to form micelles. Micelles are water-soluble poly-molecular aggregrates with a discoid configuration in which the polar groups of the lipid molecules are at the surface projecting into the aqueous medium, while the apolar hydrocarbon parts are at the core. Bile salt micelles desorb polar lipids from the surface of emulsified droplets, thus allowing hydrolysis to proceed [183-185]. A substantial proportion of dietary triglycerides can be absorbed even in the absence of bile salts (even up to 75%), as in biliary obstruction and diversion [186], suggesting that a mechanism of lipid solubilization can compensate for bile salt deficiency. Phospholipids for instance, are relatively independent from bile components for their efficient intestinal absorption. Phospholipids have a higher tendency than triglycerides to interact with water and can associate into liquid crystals, which have been suggested to play a role in luminal lipid solubilization under bile-deficient conditions [187].

Mucosal uptake

Lipolytic products are mainly absorbed taken up by proximal jejunal enterocytes [188]. During the process intestinal absorption, lipids solubilized in micelles must dissociate from them, and this occurs at a thin water layer adjacent to the lumenal surface of the enterocytes, the unstirred water layer [189,190]. The existence of an acidic microclimate in this water layer (pH 5.3-6.0), promotes both micellar dissociation and fatty acid protonation, facilitating diffusion

of fatty acids across the cellular lipid membrane [191]. Schoeller *et al.* identified a sodium/hydrogen exchanger in the brush border membrane of rat jejunal enterocytes, which is probably responsible for the acidification of the unstirred water layer [192]. Passive diffusion of fatty acid monomers across the microvillous membrane is very rapid [188,193].
In addition to rapid diffusion some fatty acid transport proteins have been identified. Fatty acid transport protein 4 (FATP4) and fatty acid translocase (FAT/CD36) are present on intestinal brush border membrane transporters and have been suggested to facilitate transport of fatty acids across the cellular membrane [194,195]. Recently, Nassir *et al.* postulated that Fat is important for fatty acid and cholesterol uptake by the proximal intestine but not the distal intestine, based on reduced uptake of fatty acids (50%) and cholesterol (60%) in Fat-deficient primary proximal enterocytes [196]. However, Goudriaan et al. demonstrated that lipid absorption was not affected in Fat knockout mice [197]. Targeted disruption of Fatp4 in mice was shown to be lethal. Heterozygous Fatp4 deletion mice showed decreased fatty acid uptake by enterocytes *ex vivo*, but not *in vivo*, possibly due to the large excess capacity of the small intestine for lipid absorption [198]. The subcellular localization of FATP4 has been a subject of debate. Stahl *et al.* showed significant amounts of FATP4 on the apical membrane of enterocytes [195], while Milger *et al.* found that FATP4 was localized exclusively intracellularly in enterocytes [199]. Milger *et al.* showed increased fatty acid uptake rate in case of FATP4 overexpression. However, they proposed that FATP4 may not be involved in fatty acid translocation at the plasma membrane, but may rather drive fatty acid uptake indirectly by stimulating its intracellular esterification [199].
Cholesterol uptake by the enterocyte can occur via diffusion or active transport systems [180,200-202]. Two groups identified two adjacent genes *ABCG5* and *ABCG8* encoding membrane transporters in the intestine and the liver [203,204]. Studies with transgenic and knockout mice showed that heterodimerization of ABCG5 and ABCG8 results in a functional sterol transporter, transporting cholesterol and plant sterols out of the cell across the apical membrane of enterocytes or hepatocytes. ABCG5 and ABCG8 are localized at the apical brush border membrane of enterocytes and at the canalicular membrane of hepatocytes [205-209]. Altmann *et al.* identified Niemann Pick C1 Like1 (NPC1L1) as a cholesterol uptake transporter. NPC1L1 is predominantly expressed in the intestine, with peak expression in the jejunum, corresponding with the efficiency of intestinal cholesterol absorption [210,211]. Davies *et al.* showed that NPC1L1 is localized at the apical membrane of enterocytes [212]. NPC1L1 is essential for intestinal cholesterol absorption, evidenced by the observation that Npc1l1 knockout mice are defective in intestinal cholesterol uptake [210,213].

Re-esterification and chylomicron formation
Once taken up by via the apical membrane of the enterocyte, fatty acids bind to IFABP and diffuse to the endoplasmic reticulum. Induction of cytosolic intestinal fatty acid binding protein (IFABP) expression in differentiated intestinal cells correlated with increased re-esterification, but not with enhanced fatty acid uptake [214]. In the endoplasmic reticulum fatty acids are activated to acyl-CoA and are subsequently re-esterificated into triglycerides [188]. Two major pathways exist for synthesizing diacylglycerol: the glycerol phosphate pathway and the monoacylglycerol pathway. In the glycerol pathway, which functions in most cells, diacylglycerol is derived by the dephosphorylation of phosphatidic acid produced by sequential acylations of glycerol phosphate. In the monoacylglycerol pathway, reported predominantly in the intestine, diacylglycerol is formed directly from monoacylglycerol and fatty acyl-CoA in a reaction catalyzed by acyl-CoA:monoacylglycerol acyltransferases

(MGATs) [215,216]. MGAT1 is expressed in most tissues but not the intestine. MGATs 2 and 3 are primarily expressed in the intestine and may be the key contributors to triglyceride packaging within the enterocytes [217-219]. Esterification of diacylglycerol into triglycerides is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) 1 and 2. In humans, DGAT1 is highly expressed in the small intestine and the colon [220,221]. Mice lacking Dgat1 were found to have normal plasma triglyceride levels, suggesting alternative mechanisms by which triglycerides can be synthesized [222]. DGAT2 possesses widespread expression in humans, with particularly high levels in liver and adipose tissue [223]. The expression patterns of DGAT1 and DGAT 2 indicate that they might have different functions within different tissues. DGAT1 likely plays a role in intestinal triglyceride resynthesis, whereas DGAT2 may function primarily in triglyceride synthesis and export from the liver [224]. Esterification of cholesterol into cholesteryl esters is catalyzed by acyl-CoA cholesterol acyltransferase 2 (ACAT2). ACAT2 expression is limited to the liver and small intestine [225]. The importance of ACAT2 in cholesteryl ester synthesis was evidenced by the observation that Acat2 knockout mice have impaired dietary cholesterol absorption and are resistant to diet-induced hypercholesterolemia and gallstone formation [226,227]. Microsomal triglyceride transfer protein (MTTP) is responsible for the assembly of cholesterol, triglycerides and phospholipids together with one apolipoprotein ApoB48 molecule (among other lipoproteins) to form a chylomicron particle, and with one ApoB100 molecule (among other lipoproteins) to form a very low density lipoprotein (VLDL) particle [228,229]. Humans only have ApoB48 in the intestine, thus VLDL particles of intestinal origin contain ApoB48. Phospholipids of luminal origin are predominantly used by enterocytes for the assembly of the surface coat of chylomicrons [230,231]. Mice with a conditional intestine-specific Mttp deletion had impaired cholesterol absorption, large cytoplasmic triglyceride droplets and no chylomicron-sized particles [232], stressing the significance of MTTP in chylomicron formation. Moreover, Wetterau et al. demonstrated that a defect absence of MTTP is responsible for the disorder abetalipoproteinemia, characterized by a defect in assembly or secretion of VLDLs and chylomicrons [233].

VLDLs (diameter 30-80 nm, $0.93<\rho<1.006$ g/ml) are the predominant lipoproteins secreted during the fasting state [234,235]. In the postprandial state, chylomicrons (diameter 75-450 nm, $\rho<0.93$ g/ml) secretion is induced after fat digestion [236,237]. Chylomicrons and VLDLs are secreted through the basolateral membrane of enterocytes into the interstitium, enter the lymphatic capillaries of intestinal microvilli that drain into omental lymphatic channels, eventually reaching the systemic circulation through the thoracic duct [188].

Lipoprotein metabolism

Once entered the circulation, chylomicrons and VLDLs interact with other lipoproteins such as LDL and HDL. Lipid transfer between these plasma lipoproteins is mediated by plasma lipid transfer proteins, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) [238].

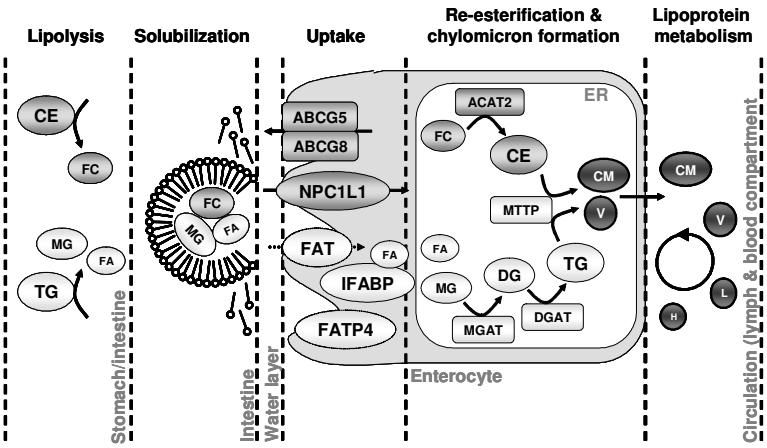

Figure 4 Schematic image of the specific phases of lipid absorption. CE = cholesteryl ester, FC = free cholesterol, TG = triglyceride, DG = diacylglycerol, MG = monoacylglycerol, FA = fatty acid, CM = chylomicron, V = VLDL, L = LDL, H = HDL.

ESSENTIAL FATTY ACIDS

The essential fatty acids (EFAs), linoleic acid (LA) and α-linolenic acid (ALA), are not synthesized *de novo* and thus must be derived from the diet. Since EFAs are abundantly available in the normal diet, EFA deficiency is relatively rare in humans. LA can be elongated and desaturated to dihomo-γ-linolenic acid (DGLA) and arachidonic acid (AA), and ALA to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EFAs are polyunsaturated fatty acids (PUFAs), which are characterized by a long carboxyl chain and the presence of double bond. There are four independent families of PUFAs, depending on the parent fatty acid from which they are synthesized. The ω-3 series is derived from ALA (18:3ω-3), the ω-6 series from *cis*-LA (18:2ω-6), the ω-9 series from oleic acid (OA; 18:1ω-9) and the ω-7 series is derived from palmitoleic acid (PA; 16:1ω-7). The first number designates the amount of carbon atoms, the second number designates the amount of double bonds, and the (ω-X) or (n-X) indicates position of the double bond carbon closest to the methyl end. The latter two series are not EFAs, since they can be synthesized in mammals.

EFAs and their metabolites have been shown to affect cell membrane fluidity, induce second messenger action and exhibit antibiotic actions. Cell membrane fluidity is determined by its lipid composition among other factors. Increased incorporation of saturated fatty acids and cholesterol into the cell membrane phospholipids renders the cell membrane more rigid, while increased incorporation of unsaturated fatty acids will make it more fluid [239-241]. Moreover, EFA metabolites are precursors to prostaglandins, thromboxanes, leukotrienes, lipoxins and resolvins. EFAs and EFA-derived compounds have been shown to play significant roles in the pathobiology of many disorders, including collagen vascular diseases, hypertension, diabetes mellitus, metabolic syndrome, psoriasis, eczema, atopic dermatitis, coronary heart disease, atherosclerosis and cancer [239]. **Figure 5** shows an overview of EFAs and their metabolites.

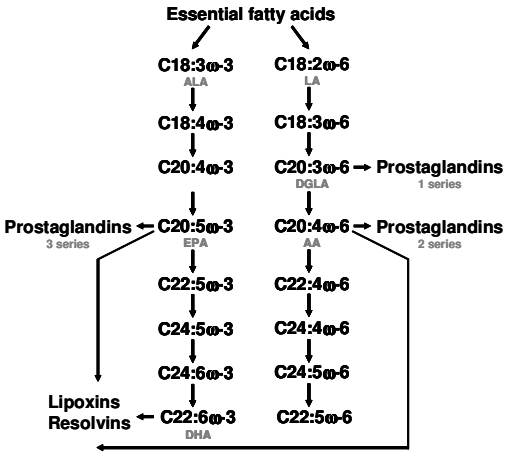

Figure 5 Dietary essential fatty acids and their metabolites.

FAT MALABSORPTION IN CHOLESTASIS AND EFA DEFICIENCY

High incidence of fat malabsorption and EFA deficiency has been reported in cholestatic patients [1,97,242-244]. Fat malabsorption is defined by an intestinal absorption efficiency lower than 95% of the amount ingested via the diet [188]. Interestingly, EFA deficiency can not only be caused by fat malabsorption, it can also itself induce fat malabsorption [243,245,246]. For reasons of clarity, fatty acid absorption will be referred to as fat absorption.

Previous studies in our laboratory revealed that both cholestasis and bile-deficiency are associated with impaired plasma LA status in relevant rat models (bile duct ligation and permanent bile diversion, respectively) [247,248]. Interestingly, impaired plasma LA status in cholestatic rats was due to decreased net absorption [247], while in bile-deficient rats posabsorptive metabolism of LA (increased plasma AA) was increased [248].

The mechanism underlying EFA deficiency-induced fat malabsorption has been extensively studied, but has not been entirely elucidated yet. Fat digestion and fat uptake were not affected in EFA deficient rats [249,250]. Levy *et al.* demonstrated that bile flow and biliary secretion of bile salts, phospholipids and cholesterol were significantly decreased in EFA deficient rats [250,251]. In addition, intracellular events such as re-esterification and chylomicron production were impaired during EFA deficiency in rats [249,250]. Thus, EFA deficiency-induced fat malabsorption in rats can be ascribed to both intraluminal (bile formation) and intracellular events (fat processing). In contrast to rats, however, EFA deficiency in mice was associated with increased bile flow and biliary secretion of bile salts, phospholipids and cholesterol [252].

Biliary phospholipids can stimulate dietary fat absorption by facilitating intraluminal lipid solubilization and by providing surface components for chylomicron assembly. Biliary secretion of phospholipids with a different composition could theoretically be involved in EFA deficiency-induced malabsorption in mice [253]. Voshol *et al.* showed that postprandial appearance of chylomicrons was impaired in mice deficient for phospholipid floppase Mdr2. Net absorption of dietary lipids, however, was unchanged in *Mdr*$^{-/-}$ mice [254]. Werner *et al.* demonstrated that absence of biliary phospholipid secretion (*Mdr2*$^{-/-}$) did not change fat malabsorption in EFA deficient mice, compared with control (*Mdr2*$^{+/+}$) EFA deficient mice.

[252]. Werner *et al.* also demonstrated that absence of biliary phopsholipid secretion (*Mdr2*$^{-/-}$) increased chylomicron size, while enhanced biliary phospholipid secretion (EFA deficient) yielded smaller chylomicrons in mice lymph [253], further emphasizing the importance of biliary phospholipid secretion in fat absorption.

Some pathological changes have been noted in the intestine of EFA deficient rats, i.e. a restricted surface area due to villi shortening and a lack of cellular differentiation [255]. This effect has been ascribed to decreased EFA content of biliary phosphatidylcholine found in EFA deficient rats. Acyl chain analysis of biliary phosphatidycholine revealed strongly decreased LA and AA content, compensated by increased oleic acid and palmitoleic acid content [249,250]. The concentration and composition of fatty acids in biliary phosphatidylcholine was also found to affect chylomicron assembly, secretion and clearance [256,257].

In normal diets, 90% of EFAs are present as acyl esters in triglycerides (90%), and only 10% as acyl esters in phospholipids and cholesteryl esters. Biliary phospholipids can facilitate dietary lipid absorption under bile-deficient conditions (e.g. cholestasis) [258]. Indeed, oral EFA supplementation in the form of phospholipids was found more effective than in the form of triglycerides in increasing LCPUFA concentrations in liver and brain of cholestatic EFA deficient mice [259].

INTESTINAL CARBOHYDRATE DIGESTION AND ABSORPTION

Besides lipids, dietary carbohydrates serve as an important source of energy. **Figure 6** shows a schematic overview of disaccharidase digestion and absorption. Dietary carbohydrates include polysaccharides (starches) and sugars (di- and monosaccharides). Starches from plants make up 75% of dietary carbohydrates and are composed of amylase (linear α-1-4-linked D-glucose polymer) and amylopectin (linear α-1-4-linked D-glucose polymer, with additional α-1-6 linkages). Intestinal digestion commences with salivary and pancreatic amylases. Alpha-amylase produces linear maltose (glucose-α-1-4-glucose) and isomaltose (glucose-α-1-6-glucose) oligosaccharides as well as some large oligomers. Final hydrolysis occurs in the small intestine where brush-border membrane maltase-glycoamylase and sucrase-isomaltase hydrolyze oligosaccharides to glucose, which is subsequently taken up by the sodium-dependent glucose transporter SGLT1. Sucrase-isomaltose digests all of the sucrose and 80% of the dietary maltose, while maltase-glycoamylase digests the remaining maltose and glucose oligomers. Dietary sugars (disaccharides) include lactose (milk), sucrose (sugar beet and sugar cane) and minor amounts of trehalose (mushrooms). These sugars are hydrolyzed by intestinal brush border membrane disaccharidases; lactase, sucrase and trehalase. Lactose is hydrolyzed into glucose and galactose, while hydrolysis of sucrose yields glucose and fructose. Dietary monosaccharides (mostly derived from fruits) are transported into the enterocytes directly by brush-border membrane transporters. Fructose is transported by the uniporter glucose transporter GLUT5 and glucose and galactose by SGLT1. Transport into the circulation of all monosaccharides occurs via GLUT2 [202,260]. Regulation of sucrase and lactase gene transcription has been shown to be exerted by cooperative action of transcription factors HNF1α, GATA4 and CDX2 [261-264].

It is rather unexplored whether cholestasis affects intestinal carbohydrate digestion and absorption. Borges *et al.* reported that obstructive jaundice did not affect jejunal absorption of the monosaccharide glucose in rats [265]. In addition, sucrase enzyme activity was shown to

be unaffected in cholestyramine-fed and bile-diverted rats, based on determinations in intestinal tissue samples [266].

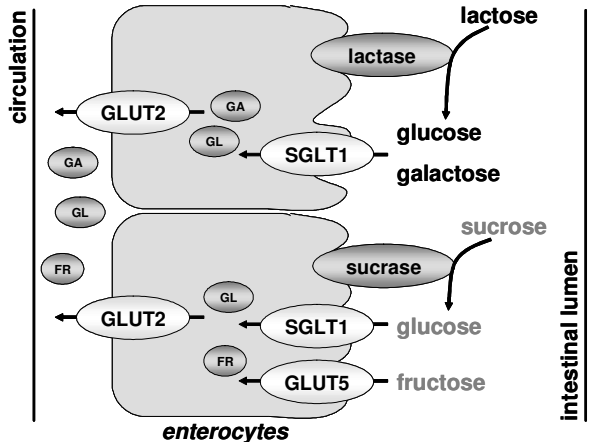

Figure 6 Schematic image of enterocytes digesting lactose and sucrose and subsequently absorbing the hydrolysis products; monosaccharides glucose

FARNESOID X RECEPTOR

As stated before, FXR is involved in the regulation of the enterohepatic circulation of bile salts and the feedback regulation of bile salt synthesis. FXR activation has been suggested to serve as a protective mechanism, because potential toxicity of bile salt accumulation, evident in cholestatic disorders, is counteracted by bile salt-activated FXR [267]. Three groups simultaneously identified bile salts as ligands for FXR [69-71]. There are two known FXR genes, commonly referred to as *FXRα* and *FXRβ*. In humans and mice, *FXRα* encodes four isoforms, FXRα1-4, result from different promoters and transcription intitation sites as well as alternative splicing of the RNA [268,269]. *FXRβ* encodes a functional member of the nuclear receptor family in rodents, rabbit and dogs, but is a pseudogene in humans and primates [270]. FXRα is mainly expressed in the liver, gut, kidney and adrenal glands, with much lower levels in adipose tissue [268,269,271,272]. Many FXR target genes are regulated in an isoform-independent manner, however, IBABP and fibroblast growth factor 19 (FGF19) are more responsive to the FXRα2 and FXRα4 [269,273,274]. FXR isoforms modulate expression of genes by binding either as a monomer or as a heterodimer with retinoid X receptor (RXR) to DNA sequence motifs [267]. FXR isoforms can be activated by structurally different ligands, including several primary and secondary bile salt species conjugated to either taurine or glycine, CDCA being the most potent ligand [69,71]. The ligand binding domain is not conserved between species. For instance, mouse FXR is more responsive to CA than its human counterpart [275]. In the next sections, the role of FXRα (referred to as FXR) in bile salt metabolism, lipid metabolism and glucose metabolism will be discussed.

FXR in control of bile salt metabolism

During the last decade, it has become clear that FXR has a crucial role in regulating bile salt metabolism. FXR regulates expression of genes involved in (1) bile salt synthesis, (2) hepatic secretion of bile salts and (3) phospholipid into the bile, (4) remaining (apart from hepatic secretion) bile salt transport, and (5) bile salt conjugation and (subsequent) bile salt

detoxification [276]. **Figure 7** is a schematic image of the effect of bile salt-induced FXR activation on its target genes involved in bile salt metabolism.

(1) Expression and induction of Cyp7a1 in rodents are dependent on oxysterol-activated liver X receptor α (LXRα) and liver receptor homolog 1 (LRH1) [277]. The human CYP7A1 gene, in contrast, lacks a LXRα response element [278]. FXR activation induces expression of small heterodimer partner (SHP) [279,280], a member of the nuclear receptor family that lacks a DNA-binding domain [281]. SHP, in turn, can dimerize with and inactivate both LXRα and LRH1, resulting in a decrease in Cyp7a1 expression [279,280,282]. Support for this regulatory cascade comes from studies showing that treatment of Shp knockout mice with a potent, synthetic FXR agonist (GW4064) fails to repress Cyp7a1 [283,284]. Another pathway that regulates bile salt synthesis is initiated after activation of FXR in enterocytes. This activation results in enhanced transcription and secretion of fibroblast growth factor 15 (Fgf15; FGF19 in humans) [285]. Subsequent binding of Fgf15 to fibroblast growth factor receptor 4 (FGFR4) on the hepatocyte cell membrane results in the activation of the JNK pathway and repression of Cyp7a1 and Cyp8b1 [285].
Moreover, FXR was found to induce expression of intestinal fibroblast growth factor 19 (FGF19; Fgf15 in rodents), a secreted growth factor that signals through the FGFR4 cell-surface receptor tyrosine kinase. FGF19/Fgf15 strongly suppresses expression of CYP7A1 in primary cultures of human hepatocytes and mouse liver through a JNK-dependent pathway [286]. Mice lacking Fgf15 have increased hepatic CYP7A1 mRNA, protein levels and enzyme activity [285].

(2) Bile salt-activated FXR activates transcription of the canalicular bile salt export pump BSEP, and thus facilitates bile salt excretion into the bile [287]. Also MRP2 expression was induced upon FXR activation [288].

(3) Huang *et al.* demonstrated that FXR activation by CDCA and GW4064 induces expression of the human MDR3 gene [289]. The mouse MDR3 homolog Mdr2 was also shown to be induced upon FXR activation [290]. Induction of MDR3/Mdr2 expression by bile salt-activated FXR increases phospholipid excretion into bile.

(4) Negative feedback regulation rat Ntcp by bile salt-activated Fxr has been shown to occur via Shp induction, leading to decreased transport of bile salt into the hepatocytes [291]. Ileal expression of ASBT in the rat is unaffected by bile salts, while in humans and mice it is under negative feedback regulation via a FXR mediated, SHP dependent effect [292]. Thus, bile salt-activated FXR reduces the amount of bile salts reabsorbed in cholangiocytes and terminal ileal enterocytes in humans and mice. In contrast to ABST, human IBABP was shown to be induced upon FXR activation *in vitro* and *in vivo* [293]. Ballatori *et al.* demonstrated that bile salt transporter OSTα/β is localized to the basolateral membrane in humans and rodents [40]. Apical to basolateral transport of TCA was shown to be increased by expression of Ostα/β in polarized canine kidney cells, while basolateral to apical transport was unaffected [294]. This indicates that OSTα/β transports bile salts out of the cell. Human and mice OSTα/β was shown to be positively regulated by bile salts via FXR [274,295]. Strikingly, Frankenberg *et al.* found both Fxr response elements and a Lrh1 response element in the mouse Ostα and Ostβ promoters. Mouse Ostα/β is thus positively and negatively regulated by bile salts. Although the positive regulatory pathway appears to be dominant, this arrangement provides a mechanism to finely titrate Ostα/β expression to the bile salt flux [295].

(5) FXR is also involved in the regulation of bile salt conjugation with glycine or taurine, evidenced by the observation that Bacs (bile acid CoA synthethase) and Baat (bile acid-

CoA:amino acid *N*-acetyltransferase) are induced by Fxr in the rat liver. Furthermore, Bacs and Baat mRNA levels were upregulated by treatment with the Fxr agonist GW4064 in rats [296], facilitating bile salt conjugation. SULT2A1 (dehydroepiandrosterone-sulfotransferase) is a cytosolic enzyme that mediates sulphate conjugation of bile salts. SULT2A1 is expressed in the liver and the intestine, and its expression has been shown to be increased upon FXR activation by CDCA. Song *et al.* speculated that increased solubility of sulphated bile salts facilitates their intracellular transport and clearance [297]. Finally, expression of human UGT2B4 (uridine 5'-disphosphate-glucuronosyltransferase 2B4), which catalyzes the conversion of hydrophobic bile salts into more hydrophilic glucuronide derivatives, was increased upon CDCA and GW4064 induced FXR activation [298].

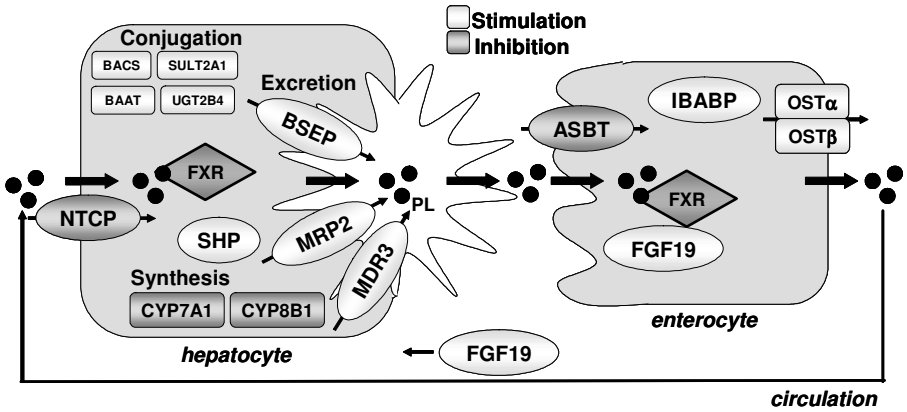

Figure 7 Schematic image of stimulatory and inhibitory actions of FXR with respect to bile salt homeostasis [adapted from 68].

FXR in control of lipid metabolism

The involvement of bile salts in the regulation of lipid metabolism became apparent when treatment of patients suffering from gallstones or hypertriglyceridemia with CDCA reduced plasma triglycerides [299,300]. Some important FXR actions related to lipid metabolism are listed below. Sinal *et al.* demonstrated that FXR is involved in the control of plasma lipid levels, evidenced by increased plasma levels of triglycerides and cholesterol in *Fxr*$^{-/-}$ mice [301]. *Fxr*$^{-/-}$ mice were also shown to have higher levels of HDL cholesterol, consistent with reduced expression of SR-B1, the scavenger receptor that facilitates clearance of HDL cholesterol from blood [302,303]. Moreover, administration of FXR agonists to healthy rats and mice reduced plasma triglyceride levels [304,305]. FXR activation also alters transcription of genes involved in fatty acid and triglyceride synthesis and lipoprotein metabolism [306]. Bile salt activated Fxr led to lower plasma triglyceride levels via induction of Shp and subsequent repression of mouse Srebp-1c, a transcription factor that controls genes involved in fatty acid and triglyceride synthesis [307]. These decreased levels of plasma lipids were associated with decreased VLDL secretion, caused by reduced expression of MTTP in hamsters fed with CDCA and in HepG2 cells exposed to CDCA [308,309].

SCOPE OF THIS THESIS

As stated before, cholestatic liver disease is frequently associated with nutritional defects, including EFA deficiency. The resultant malnutritional state strongly affects prognosis and treatment outcome in cholestatic children. Our ultimate goal is to improve the prognosis of cholestatic children by optimization of their nutritional status. As one of the strategies to achieve this, we herewith aimed to elucidate the effects of cholestasis and EFA deficiency on intestinal function, with emphasis on nutrient absorption.

This thesis focusses on the intestinal digestion and absorption of fat and carbohydrates in cholestasis and EFA deficiency. In **chapter 2**, the nutritional status of children with cholestatic liver disease and potential treatment options are discussed. The majority of cholestatic disorders are associated with elevated plasma bile salt levels. Bile salts have been shown to induce proliferation and apoptosis in intestinal cells *in vitro*. Theoretically, high plasma bile salt levels could affect intestinal epithelial cells and their capacity to absorb nutrients. In **chapter 3**, intestinal function in cholestatic rats is compared to intestinal function in control rats to elucidate the intestinal effects of cholestasis. With stable isotope methodology intestinal digestion and absorption of sucrose and glucose were assessed. To be able to pinpoint possible effects to high plasma bile salt levels, cholestatic and control rats were compared to bile-deficient rats. In **chapter 4** the effects of cholestatic conditions on enterocytes in different developmental stages are investigated, by exposing human intestinal epithelial cells to bile salts in cholestatic concentrations *in vitro*.

Cholestasis-induced fat malaborption is often associated with EFA deficiency. EFA deficiency itself can also induce fat malabsorption. The underlying mechanism, however, has not been elucidated. Since EFA deficiency has been associated with pathological changes in small intestine, i.e., a restricted surface area due to villi shortening and a lack of cellular differentiation, intestinal absorption of other nutrients could theoretically also be affected. To test this theory, intestinal function and its capacity to digest and absorb carbohydrates will be investigated during EFA deficiency. In **chapter 5** intestinal function and digestion and absorption of lactose and glucose was assessed in EFA deficient mice.

Finally, like EFAs, the nuclear receptor FXR has been shown to be involved in bile salt homeostasis and lipid homeostasis. We speculate that FXR is involved in EFA deficiency-induced fat malabsorption. In **chapter 6** the involvement of FXR in bile formation and fat malabsorption was assessed by comparing these parameters in EFA deficient Fxr knockout mice and EFA deficient control mice.

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CHAPTER 2

Nutrition for children with cholestatic liver disease

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ABSTRACT

Cholestatic liver disease (CLD) in children negatively affects nutritional status, growth and development, which all lead to an increased risk of morbidity and mortality. This is illustrated by the fact that the clinical outcome of children with CLD awaiting a liver transplantation is in part predicted by their nutritional status, which is integrated in the Pediatric End-Stage Liver Disease model. Preservation of the nutritional status becomes more relevant as the number of patients waiting for liver transplantation increases and the waiting time for a donor organ becomes prolonged. Nutritional strategies are available to optimize feeding of children with CLD. Patients with CLD, however, form a heterogeneous group and the clinical manifestations of their disease vary. This makes a tailor-made approach for these children crucial. Not all aspects of nutrient metabolism and absorption in children with CLD are well understood and studied. Experiments with stable isotope-labeled triglycerides and fatty acids have provided essential information about fat absorption under physiological and cholestatic conditions in animal models and humans. We expect that in the future, tests using other isotope-labeled macronutrients, i.e. carbohydrates and proteins, can be used to further assess nutritional status of children with CLD thereby creating tailor-made nutritional therapies.

CHOLESTASIS AND NUTRITIONAL STATUS

Cholestatic liver disease (CLD) negatively affects nutritional status, growth and development, particularly in infancy, i.e. when growth rates are highest. The presence of malnutrition and growth retardation (failure to thrive) compromises the clinical outcome for children with end-stage liver disease. Cholestatic children with a poor nutritional status, who require liver transplantation have an increased risk of morbidity and mortality [1,2]. To illustrate this, the nutritional status is an important contributor to Pediatric End-stage Liver Disease (PELD) score for children under 2 years of age. The PELD score is a reliable predictor of mortality in children with CLD on the waiting list for liver transplantation. The PELD model was implemented by the United Network of Organ Sharing (UNOS) in the United States in February 2002 as an improved algorithm for allocating livers among pediatric orthotopic liver transplant candidates, and will be adopted by the Eurotransplant society in January 2007. It has recently been shown that the PELD score also correlates with posttransplant survival [3]. Preservation of the nutritional status becomes more relevant as the number of patients waiting for liver transplantation increases and the waiting time for a donor organ becomes prolonged.

NUTRITION FOR CHOLESTATIC CHILDREN

Poor dietary intake is an important factor in the pathophysiological basis of malnutrition in children with CLD. Furthermore, their nutritional status may be further compromised by decreased absorption of macronutrients, including fat, carbohydrates and proteins [4]. At an early age, fat accounts for the most important dietary energy source (up to 50% of total ingested energy). Essential fatty acids (EFA) and long-chain polyunsaturated fatty acids (LCPUFA) are indispensable for proper development and function of different organs, for example the central nervous system. Micronutrient absorption may also be affected in CLD, including absorption of fat-soluble vitamins A, D, E and K.

The dietary prevention or treatment of failure to thrive during CLD involves some general principles applicable to virtually all patients and some more individual tailor-made approaches. Nutrition in infancy consists predominantly of breast milk or formula. For children with CLD, the dietary energy intake is usually increased to levels of 120-150% of recommended daily energy intake (corrected for age and gender). The adaptation of the formula diet usually involves increasing the concentration and amount ingested. In addition, up to 60% of the fat components, particularly long-chain triglycerides are substituted by medium-chain triglycerides (MCTs), whose absorption can occur relatively independently from the presence of bile components in the intestinal lumen. The carbohydrate content can be increased by supplementation of formula with maltodextrin. Breastfed children receive additional formula and MCT-rich oil, while for older children feeding with formula is often prolonged and energy-rich liquids are provided. Adequate absorption of fat-soluble vitamins during CLD can usually be obtained by considerably increasing the dosages administered daily, well above regular recommendations for the age groups. Serum levels of fat-soluble vitamins are regularly monitored, in order that dosages can be adapted. Adequate intake of EFA and LCPUFA is not frequently monitored in CLD patients, but should be reached when these fatty acids are provided in ample amounts in the diet. Nevertheless, we reported that ~70% of children with CLD requiring liver transplantation have biochemical indications of EFA and LCPUFA deficiency [5].

Reduced gastric volume, vomiting, ascites and hypoglycemia lead to limited absorption of the required dietary nutrients when administered in regular (bolus) feedings. Under these circumstances, continuous nasogastric drip feeding may be needed to guarantee maximal uptake of nutrients.
For some time now, a special formula for infants with CLD has been available. The composition of this formula aimed to accommodate the general aspects of nutritional support needed for infants with CLD. So far, however, no data have become available to substantiate its benefit, nor its advantage over conventional dietary treatment: supplementation of MCT-rich formulas with carbohydrates, fat-soluble vitamins and EFA. Clinical data are needed to determine the role that this formula can play in the dietary treatment of infants with CLD.

BIOLOGICAL ASPECTS OF NUTRITION FOR CHOLESTATIC CHILDREN

Cholestatic diseases in children
CLD is characterized by decreased or absent hepatic secretion of bile into the intestine. The most common cause of CLD in children requiring liver transplantation is biliary atresia. Biliary atresia is a progressive disorder characterized by an inflammatory reaction towards the extrahepatic and intrahepatic bile ducts, leading to their destruction and subsequent replacement by fibrotic scar tissue. The etiology of biliary atresia remains unknown, although an inflammatory reaction to a detrimental stimulus seems to play an initiating role. Suggested initiating stimuli include specific perinatal viral infections, genetic factors, defects in immune response, as well as defects in morphogenesis. Another disease that can lead to end-stage liver disease in infancy is Alagille's syndrome, an autosomal dominantly inherited syndrome including bile duct hypoplasia, and congenital anatomical defects in other organs. Progressive Familial Intrahepatic Cholestasis (PFIC) is also a genetically transmitted disorder, but is inherited in an autosomal recessive fashion. Three phenotypic forms of PFIC have been characterized and attributed to gene defects in three different genes (PFIC1-3). Another cause of CLD is nonsyndromic paucity of the intrahepatic bile ducts, which is suggested to be the result of various infections, chromosomal disorders or metabolic disorders. Finally, inborn errors in bile acid synthesis account for part of the children with CLD. Defects have been identified in enzymes catalyzing cholesterol catabolism and bile acid synthesis [6].
CLD in adolescents and young adults is often due to autoimmune hepatitis, primary biliary cirrhosis or primary sclerosing cholangitis [6,7].

Although the causes and clinical manifestations of CLD may vary, it is often accompanied by liver damage. The obstruction or absence of bile ducts leads to accumulation of bile acids in hepatocytes, which results in liver damage. Because the enterohepatic circulation of bile acids is interrupted, the resulting absence of bile acids in the intestinal lumen leads to impaired micellization and therefore to strongly reduced absorption of fats and fat-soluble nutrients. Another feature of CLD is the high serum bile acid level, which can cause secondary tissue injury.
In biliary atresia, it is often attempted to correct the enterohepatic circulation of bile acids by performing a Kasai portoenterostomy. During this procedure the liver is directly connected to the proximal small intestine to optimize bile flow into the intestine as much as possible.

However, Kasai portoenterostomy is frequently only a transient solution, due to the presence of intrahepatic bile duct damage and ongoing liver damage. Most patients with biliary atresia eventually need liver transplantation. As is pointed out above, the nutritional status of children with CLD is important for the clinical outcome of liver transplantation and for long-term survival after liver transplantation. Besides the obviously reduced absorption of fats and fat-soluble vitamins, chronic cholestasis also affects dietary intake, energy metabolism and metabolism of macronutrients as well as micronutrients.

DIETARY INTAKE AND ENERGY EXPENDITURE

Dietary intake

Reduced dietary intake is an important contributor to malnutrition in children with CLD. Fatigue, anorexia, nausea, vomiting, diarrhea, altered or reduced ability to taste, and early satiety may all contribute to decreased ingestion of food. Organomegaly and ascites can further compromise dietary intake by reducing gastric capacity. Additionally, many diet modifications, for example sodium, fluid or protein restrictions, make food even more inpalatable. These dietary restrictions are imposed on patients with relatively high risks of fluid overload and encephalopathy, which, when left untreated, can lead to serious irreversible defects [8].

Energy metabolism

Energy expenditure is composed of the basal metabolic rate (BMR), the amount needed for growth and metabolism. Although clinical data are conflicting, some children with CLD have been shown to have an increased BMR. Shanbhogue *et al.* [9] reported a higher BMR, when related to lean tissue in patients with end-stage liver disease. In children with biliary atresia energy expenditure was 29% higher than healthy controls [10]. Also Shepherd [11] reported higher energy expenditure per unit body cell mass in children with biliary atresia In contrast, Muller *et al.* [12] found that patients with cirrhosis showed a variable BMR, in the range from hypometabolic to hypermetabolic Another study showed an unchanged BMR in children with Alagille syndrome [13]. A hypermetabolic state could be an important factor in the clinical outcome for CLD, because it further aggravates nutritional status.

Recently, Watanabe *et al.* [14] found that bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. In this study, mice were fed a bile acid (cholic acid)-containing high fat diet. These mice showed subsequent reduction in weight gain, white adipose tissue weight and brown adipose tissue weight compared to mice on a high fat diet. In addition, animals fed a high fat diet containing cholic acid had higher CO_2 production and O_2 consumption, indicating a higher level of energy expenditure. Human skeletal muscle myocytes showed an increase in O_2 consumption after treatment with bile acids It is presently unknown, however, whether bile acid accumulation in patients with CLD is partly responsible for increased energy expenditure.

NUTRIENT METABOLISM

Apart from reduced intraluminal bile acid concentrations, other consequences of CLD, such as gastrointestinal bleeding, impaired digestive enzyme production and secretion, mucosal congestion, villous atrophy, bacterial overgrowth or pancreatic insufficiency, can lead to

maldigestion and malaborption of nutrients. In addition, even certain medications can aggravate malabsorption. For example, cholestyramine binds to bile acids in the intestinal lumen and thereby further reduces absorption of fat-soluble nutrients. Also, reduced availability of specific nutrients involved in digestion and/or absorption of other nutrients, specifically vitamins and minerals, affects intestinal absorption [8]. In the remaining part of this article we will focus on metabolism of fat, carbohydrates, protein and micronutrients in CLD.

Fat metabolism

CLD is characterized by malabsorption of fat. Especially long-chain triglycerides, which are digested to fatty acids and monoacylglycerols in the intestinal lumen, are poorly absorbed during cholestasis, due to their impaired micellization during bile deficiency.

The route that fat undertakes from the diet to the blood can be divided in four steps: emulsification, lipolysis (lipases), solubilization (bile) and translocation (mucosa). During lipolysis lipases catalyze the conversion of triglycerides to glycerol and fatty acids. The latter need to be solubilized by bile acids to be transported towards the vicinity of the mucosa. Here, at the unstirred water layer, the micelles disintegrate, after which fatty acids and monoacylglycerols are taken up across the apical membrane of the mucosal cells. Inside the enterocytes, the absorbed lipids are reacylated to triacylglycerols, assembled into chylomicrons, which are then secreted into lymph and subsequently appear in the circulation.

In our laboratory, Kalivianakis *et al.* [15] developed a stable isotope test to quantify lipolysis and absorption of long-chain fatty acids in rats. We determined absorption and appearance in plasma of ^{13}C-labeled palmitic acid in rats with malabsorption either due to chronic bile deficiency (permanent bile diversion as developed in our laboratory by Kuipers *et al.* [16]) or due to oral administration of the lipase inhibitor Orlistat. These models were used to discriminate between potential causes of fat malabsorption such as impaired intestinal lipolysis or reduced uptake of fatty acids. Rats were given a high fat diet (35% of total energy) compared to a low fat diet (14% of total energy) to potentiate the effect of fat malabsorption. Results were compared with the percentage absorption of ingested fat determined by fat balance. As expected, dietary fat absorption was significantly impaired in bile-deficient animals as compared to controls (Fig 1D). However, the net fat uptake (Fig 1C), defined as the difference between the amount of fat ingested (Fig 1A) and the amount of fat excreted ('lost') via the feces (Fig 1B) through fat malabsorption, was not significantly affected by the presence or absence of intestinal bile (control *vs.* chronic bile diversion), or the amount of fat in the diet (high fat *vs.* low fat). The percentage of total ingested fat absorbed in bile-deficient rats was only 87% with the low fat diet and 54% with the high fat diet (Fig 1D). Apparently, bile deficieny (without cholestasis) increases the nutrient ingestion in rats, compensating sufficiently for the increased energy and fat loss via the feces. Bile deficiency due to bile diversion in rats led to a decreased concentration of plasma ^{13}C-palmitic acid, indicating impaired absorption of long-chain fatty acids. Control experiments showed that lipolysis was not affected in bile-deficient rats. Impairment of fat absorption due to Orlistat had no effect on plasma ^{13}C-palmitic acid, indicating the specificity of the test [15]. This test can also be utilized to generate clinical data as was demonstrated by Rings *et al.* [17]. Rings *et al.* showed that the absorption of free fatty acids, but not fat digestion was rate-limited in neonates, and developed to adult competence within 2 months after term age. Neonates are known to have a mild 'physiological' cholestasis during the first months of life, and this may be the mechanism underlying this observation.

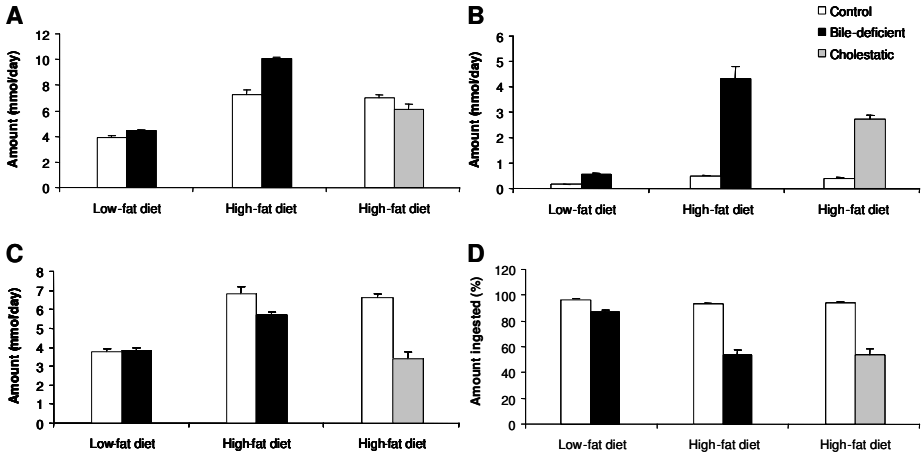

Figure 1 Fat absorption in bile-deficient and cholestatic rats on low fat and high fat diet, with (A) fat intake, (B) fecal fat excretion, (C) net fat uptake, and (D) dietary fat absorption as % of amount ingested. [adapted from 15 and 18].

EFA and LCPUFA are crucial for normal development and function. They cannot be synthesized endogenously and therefore must be provided by the diet. As is reviewed by Sealy *et al.* [5], the percentage of n-3 and n-6 fatty acids is reduced in pediatric cholestasis. This observation reflects the inability of CLD patients to absorb sufficient amounts of EFA and LCPUFA, due to absence of bile in the intestinal lumen in combination with frequently compromised dietary intake. An important consequence of this inability to acquire sufficient amounts of LCPUFA is EFA deficiency. The quantitatively most important EFAs are linoleic acid (LA) and α-LA, members of the n-6 and n-3 family of fatty acids. Linoleic and linolenic acid are precursors for LCPUFAs, including arachidonic acid (C20:4n-6), eicosapentaenoic acid (C22:5n-3), and docosahexaenoic acid (C22:6n-3), respectively.

Minich *et al.* [18] investigated fat malabsorption as a possible cause of EFA deficiency in rats that were intestinally bile deficient due to either permenant bile diversion or to bile duct ligation (cholestasis). Absorption of the EFA LA was quantified by fat balance and by measuring plasma concentrations of [^{13}C]-LA after its intraduodenal administration. Plasma concentration of [^{13}C]-LA was decreased in bile-diverted rats, while net absorption of LA from the intestine was unaffected. The fact that net absorption of fat and LA was not affected in bile-deficient rats corresponded with increased food intake (see above) in addition to relative preservation of EFA absorption under bile-deficient conditions, in comparison with nonessential saturated fatty acids, such as palmitic acid (C16:0) and stearic acid (C18:0). In cholestatic rats, however, both plasma concentration and net absorption of unlabeled or [^{13}C]-labeled LA were decreased. Metabolism of LA into arachidonic acid was not affected, indicating that LA deficiency in these rats is due to decreased net absorption. The compensatory increase in nutrient ingestion during intestinal bile deficiency on the basis of permanent bile diversion (see above) did apparently not occur in rats with intestinal bile deficiency on the basis of bile duct ligation. It is tempting to speculate that the accumulation of bile products during the latter cholestatic condition abolishes the compensatory mechanism of increased nutrient ingestion. This observation is in accordance with clinical experience that nutrient intake and appetite are compromised in children with CLD.

Children with CLD are sometimes given a fat-restricted diet, since effects such as steatorrhea/diarrhea are expected. According to the study of Kalivianakis *et al.* [15] the amount and fraction of dietary fat lost via the feces is indeed significantly less on a low fat than on a high fat diet (Fig 1B). However, net fat uptake from a high fat diet was almost 2-fold higher than that from a low fat diet (Fig 1C). The net absorption reflects the amount of nutrients (fat) that actually becomes available for energy and growth needs of CLD patients. This observation underlines a clinical strategy to increase, rather than to restrict the amount of fat intake in patients with CLD, even at the expense of steatorrhea, in order to maximize their nutritional condition.

Carbohydrate metabolism

In children with CLD, carbohydrate homeostasis can be affected by hepatic failure itself, for example by a decreased capacity of gluconeogenesis. Frequently also peripheral utilization of glucose is reduced, which may decrease the risks of hypoglycemia. In CLD, hepatic degradation of insulin may also be decreased, which may be one of the causes for the 2-fold higher insulin response in CLD compared to control patients. Elevated plasma levels of insulin in combination with glucose tolerance imply insulin resistance, which could be further aggravated by increased circulating free fatty acids as seen in CLD [8].

Apart from the hepatic effects on glucose homeostasis, the intestinal carbohydrate absorption could also be affected by CLD. However, no data are yet available about carbohydrate absorption under cholestatic conditions. Recently our group has started to investigate intestinal carbohydrate absorption under cholestatic conditions *in vitro* and *in vivo*. The underlying hypothesis for this research is that cholestasis, i.e. high serum bile acid concentrations could alter intestinal function by affecting proliferation, differentiation and/or apoptotic cell death of the absorptive intestinal epithelium [19]. These effects of cholestatic bile acid concentrations on other cell types have been well delineated. For example, relatively low concentrations of bile acids induce apoptosis in hepatocytes [20].

Protein metabolism

The catabolic reduction in total body protein as seen in CLD is mainly due to extensive liver damage. Tavill [21] and McCullough and Glamour [22] found no significantly changed protein turnover in CLD patients. Amino acid oxidation is normal or reduced in these patients, consistent with appropriate adaptation to reduced nutrient supplies. Due to hepatic insufficiency occurring in later stages of CLD, oxidation of aromatic amino acids is reduced as is the metabolism of branched-chain amino acids. Mager *et al.* [23] reported an increased dietary need for branched-chain amino acids in children with mild-to-moderate chronic CLD, due to increased postabsorptive leucine oxidation. In general, amino acids seem to be conserved in CLD, probably due to the body's ability to increase protein synthesis and reduce amino acid oxidation. This increased protein synthesis is, however, at the cost of muscle proteolysis [21,22]. Increased protein oxidation resulted in a virtually zero nitrogen balance in children with biliary atresia and even in oxidation of endogenous proteins [10]. Stable experimental conditions do not necessarily reflect the spectrum of clinical conditions frequently encountered, such as episodes of metabolic stress or infections that can increase protein turnover and catabolism [8]. Interestingly, Sokal *et al.* [24] found that branched chain amino acids improve body composition and nitrogen balance in a rat model of extrahepatic cholestasis.

Overall, addition of proteins, and especially specific amino acids such as branched chain amino acids, could improve nutritional status of children with CLD. However, care must be taken, because an excess of protein can negatively influence encephalopathy.

Micronutrient metabolism

The absence of bile acids in the intestinal lumen as observed in cholestasis reduces absorption of fat-soluble vitamins A, D, E and K, as briefly described above. Calcium uptake is at risk as a result of formation of nonsoluble calcium fatty acid soaps during fat malabsorption. Hypovitaminosis D may increase renal loss of phosphate and hypovitaminosis A may induce zinc deficiency. Zinc deficiency has a negative impact on cognitive function, appetite and taste, immune function, wound healing and protein metabolism. In addition, zinc deficiency has frequently been associated with EFA deficiency [25]. Finally, uptake of selenium can be disturbed due to EFA deficiency and iron depletion is seen as a result of gastrointestinal bleeding, insufficient uptake, transport and handling of iron. In addition, liver dysfunction strongly reduces storage capacity of vitamins such as folate, riboflavin, nicotinamide, pantothenic acid, pyroxidine, vitamin B_{12}, thiamine and vitamin A. Hepatocellular injury in CLD also results in defects in vitamin activation, conversion, release and transport [8].

As described above, children with CLD receive higher dosages of fat-soluble vitamins. Furthermore, addition of zinc to the diet could counteract a part of the poor dietary intake.

CONCLUDING REMARKS

Influencing nutritional intake and metabolism is a critical aspect of the management of children with CLD. Patients with CLD form a heterogeneous group and the clinical manifestations of their disease vary. This makes a tailor-made approach for these children crucial. Not all aspects of nutrient metabolism and absorption in children with CLD are well understood and studied. Experiments with stable isotope-labeled triglycerides and fatty acids have provided essential information about fat absorption under physiological and cholestatic conditions in animal models and humans. We expect that in the future, tests using other isotope-labeled macronutrients, i.e. carbohydrates and proteins, can be used to further assess nutritional status of children with CLD, thereby creating tailor-made nutritional therapies.

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CHAPTER 3

Intestinal capacity to digest and absorb carbohydrates is maintained in a rat model of cholestasis

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ABSTRACT

Cholestasis is associated with systemic accumulation of bile salts and by deficiency of bile in the intestinal lumen. During the past years bile salts have been identified as signaling molecules that regulate lipid, glucose and energy metabolism. Bile salts have also been shown to activate signaling routes leading to proliferation, apoptosis or differentiation. It is unclear, however, whether cholestasis affects the constitution and absorptive capacity of the intestinal epithelium *in vivo*. We studied small intestinal morphology, proliferation, apoptosis, expression of intestine-specific genes and carbohydrate absorption in cholestatic (1 wk bile duct ligation), bile-deficient (1 wk bile diversion) and control (sham) rats. Absorptive capacity was assessed by determination of plasma ^{2}H- and ^{13}C-glucose concentrations after intraduodenal administration of ^{2}H-glucose and naturally-enriched ^{13}C-sucrose, respectively. Small intestinal morphology, proliferation, apoptosis and gene expression of intestinal transcription factors (mRNA levels of *Cdx-2*, *Gata-4* and *Hnf-1α*, and Cdx-2 protein levels) were similar in cholestatic, bile-deficient and control rats. The (unlabeled) blood glucose response after intraduodenal administration was delayed in cholestatic animals, but the absorption over 180 minutes, was quantitatively similar between the groups. Plasma concentrations of ^{2}H-glucose and ^{13}C-glucose peaked to similar extents in all groups within 7.5 min and 30 min, respectively. Absorption of ^{2}H-glucose and ^{13}C-glucose in plasma was similar in all groups. The present data indicate that neither accumulation of bile salts in the body, nor their intestinal deficiency, two characteristic features of cholestasis, affect rat small intestinal proliferation, differentiation, apoptosis, or its capacity to digest and absorb carbohydrates.

INTRODUCTION

Cholestasis is associated with accumulation of bile salts in the body and by deficiency of bile salts in the intestinal lumen [1]. Bile salts facilitate dietary lipid absorption in the intestinal lumen and contribute to cholesterol homeostasis [2]. More recently, bile salts have been identified as signaling molecules. Through activation of the farnesoid X receptor (FXR), bile salts regulate various aspects of glucose and lipid metabolism as well as intestinal barrier function [2,3]. Watanabe *et al.* described a role for bile salts in the regulation of energy metabolism via the G protein-coupled bile acid receptor (GPBAR1) [4]. Finally, bile salts can activate MAPK pathways, leading to proliferation or apoptosis [2].

In children, cholestatic liver disease negatively affects nutritional status, growth and development, which cannot be explained by solely the inability to absorb lipids and lipid-soluble vitamins [5-7]. Sofar, it has remained unclear to what extent cholestasis, i.e. systemic accumulation and intestinal deficiency of bile salts, affects small intestinal epithelial proliferation, differentiation or apoptosis and, consequently, absorptive capacity *in vivo*.

In the small intestinal lumen sucrose is hydrolyzed into glucose and fructose by the brush border membrane enzyme sucrase. Glucose is actively transported across the apical membranes of enterocytes by the sodium-dependent glucose co-transporter SGLT-1. The majority of glucose is passively transported from the enterocyte into the circulation by the facilitated glucose transporter GLUT-2 [8]. Intestinal sucrase-isomaltase (SI) gene transcription is regulated by the intestine-specific transcription factors Gata binding protein 4 (GATA-4), hepatic nuclear factor 1α (HNF-1α) and caudal type homeobox transcription factor 2 (CDX-2) [9].

In the present study, we investigated whether cholestasis affects small intestinal constitution and the absorptive capacity for carbohydrates in rats.

MATERIALS AND METHODS

Rats and housing

Male Wistar rats, weighing 270-300 g at the beginning of the study, were obtained from Harlan (Horst, The Netherlands). They were individually housed in Plexiglas cages (25 x 25 x 30 cm) on a layer of wooden shavings under controlled temperature, humidity and on a 12:12-h light-dark cycle. Water and chow diet (Hope Farms, Woerden, The Netherlands) were available ad libitum. All experiments were approved by the Animal Experiments Ethical Committee of the University of Groningen.

Materials

6,6–^{2}H-glucose, 98% ^{2}H was obtained from Isotec Inc (Miamisburg, OH). Isotopic purity was confirmed by GC-MS. Cane sugar (Caribbean Gold, Amstelveen, The Netherlands) was used as naturally-enriched ^{13}C-sucrose.

Surgery

All rats were equipped with permanent catheters in the jugular vein and duodenum, as described by Kuipers *et al.* [10]. Bile duct ligated (cholestatic; $n = 5$) and bile-diverted (bile-deficient; n=7) rats were compared to sham-operated rats (control; $n = 6$). The experimental model allows for physiological studies in unanaesthetized rats with bile duct ligation and diversion without the interference of stress or restraint. These models have been proven

useful to analyze intestinal absorption capacity [11-13]. After surgery, the rats were allowed to recover for 1 wk.

Experimental procedures
Feces were collected for 24 h, between day 5 and 6 after surgery. Cholestatic, bile-deficient and control rats were subjected to a combined ^{2}H-glucose /^{13}C-sucrose absorption test at 1 week after surgery. On the day of the experiment, the rats received an intraduodenal bolus of 1 mg ^{2}H-glucose and 0.25 g ^{13}C-sucrose in 1 ml water, after an overnight fast. At t = 0, 7.5, 15, 30, 45, 60, 90, 120 and 180 minutes, blood samples were taken for determination of blood glucose concentrations and plasma ^{2}H- and ^{13}C-glucose enrichments. At the end of the experiment, the rats were sacrificed and the small intestine was collected for analysis. The small intestine was flushed with ice-cold PBS and was divided into the duodenum, the jejunum and the ileum. Material was harvested for histology and gene expression. Small intestinal mucosa was scraped for the determination of enzyme activity.

Analytical methods
Plasma bile salts were determined as described previously [14]. Fat ingestion, fecal fat excretion and net fat absorption were measured and calculated as described previously [11].

Stable isotope test
Blood glucose concentrations were measured with a Lifescan EuroFlash glucose meter (Lifescan Benelux, Beerse, Belgium). The sample preparation procedure of plasma ^{2}H-glucose enrichment and plasma ^{13}C-glucose enrichment was as described by Vonk *et al.* [15]. The ^{2}H enrichment was measured by GC-MS (Trace MS, Interscience, Breda, The Netherlands) [16]. The ^{13}C/^{12}C isotope ratio measurement of the glucose penta-acetate derivative was determined by Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry (GC-C-IRMS) using a Delta Plus instrument (Thermofinnigan, Bremen, Germany) [15]. Concentrations were calculated as described by Vonk *et al.* [16].

Disaccharidase activity
Enzyme activity levels of sucrase were measured in freshly scraped intestinal mucosa as described by Dahlqvist [17]. Activity levels were normalized to protein levels, measured by the BCA method as described by the manufacturer (Pierce, Rockford, IL).

RNA isolation and measurement of mRNA levels by real-time PCR (Taqman)
mRNA expression levels in duodenum, jejunum and ileum were measured by real-time PCR, as described previously [18]. PCR results were normalized to β-actin mRNA levels. The sequences of the primers and probes are listed in Table 1.

Histology
Morphology of jejunal sections of the small intestine was assessed by haematoxylin and eosin staining of formaline-fixated material. Proliferating cells were detected by staining of nuclear Ki-67 antigen. Apoptosis was assessed by staining DNA strand breaks with TUNEL staining according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Roche, Mannheim, Germany). Crypt and villous length were determined by morphometric procedures.

Table 1. Primer and probe sequences.

Gene	GenBank	Forward Primer	Reversed Primer	TaqMan® probe
β-actin	NM_031144	AGC CAT GTA CGT AGC CAT CCA	TCT CCG GAG TCC ATC ACA ATG	TGT CCC TGT ATG CCT CTG GTC GTA CCA C
Asbt	NM_017222	ACC ACT TGC TCC ACA CTG CTT	CGT TCC TGA GTC AAC CCA CAT	CTT GGA ATG ATG CCC CTT TGC CTC T
Ibabp	NM_017098.1	CCC CAA CTA TCA CCA GAC TTC G	ACA TCC CCG ATG GTG GAG AT	TCC ACC AAC TTG TCA CCC ACG ACC T
Shp	NM_057133	ACC TGC AAC AGG AGG CTC ACT	TGG AAG CCA TGA GGA GGA TTC	TCC TGG AGC CCT GGT ACC CAG CTA GC
Gpbar1	NM_177936.1	ACT GGT CCT GCC TCC TTC TC	GCT GCA ACA CTG CCA TGT AG	TCC CTG CTT GCC AAT CTG CTG CT
Hnf-1α	NM_012669	CTC CAG CAG CCT GGT GTT GT	GAG GCC ATC TGG GTG GAG AT	CAC AGC CAC CTG CTG CCA TCC AAC
Gata-4	NM_144730.1	GAG ATG CGC CCC ATC AAG	GAC ACA GTA CTG AAT GTC TGG GAC AT	CTG TCA TCT CAC TAT GGG CAC AGC AGC TC
Cdx-2	NM_023963	GTC CCT AGG AAG CCA AGT GAA A	CTC CTG ATG GTG ATG TAT CGA CTA	CCT TCT CCA GCT CCA GCC GCT G
Sglt-1	NM_013033	GCT GGA GTC TAC GTA ACA GCA CA	GGG CTT CTG CAT CTA TTT CAA TG	TCC TCC TCT CCT GCA TCC AGG TCG
Glut-2	NM_012879	GCA TCA GCC AGC CTG TGT ATG	GCA GCA CAG AGA CAG CTG TGA	CCA TCG GCG TTG GTG CCA TCA AC
Si	NM_013061	TGT TTG GGT GAA TGA GTC AGA TG	CCC ACC ACT CGA TGG TTT G	ACT GTT AAT CCT GGC CAT ACC TCT CCA ATA A

Statistical analysis

Values represent means ± SE for the indicated number of rats per group. Using SPSS version 12.0.2 statistical software (Chicago, IL, USA), we calculated significance of differences. Significance of differences was calculated with regard to the treatments as well as the different intestinal segments. Body weight and food intake were normally distributed and tested with the One-Way ANOVA. When the One-Way ANOVA resulted in a significant difference among the groups ($P < 0.05$) the two-tailed T-Test was used to calculate differences among the treatments or intestinal segments separately. All other parameters were not normally distributed and therefore tested with Kruskal-Wallis H and subsequently with the Mann-Whitney U for differences among the treatment groups or intestinal segments when a significant difference ($P < 0.05$) was observed.

RESULTS

Characterization of the model

Plasma bile salt concentration was significantly higher in cholestatic rats compared to control and bile-deficient rats (187 ± 20 vs. 10 ± 4 and 1.0 ± 0.5 μmol/L, respectively), and significantly lower in bile-deficient rats compared to control rats (all, $P < 0.01$).

In accordance with the localization of intestinal bile salt reabsorption, expression of the apical sodium-dependent bile acid transporter (*Asbt*) and the ileal bile acid binding protein (*Ibabp*) was restricted to the ileum [19,20]. *Asbt* expression was not quantitatively changed in cholestatic or bile-deficient rats, while *Ibabp* expression was reduced in cholestatic rats (by ~35%) and significantly reduced in bile-deficient rats compared to control rats (by ~60%; $P < 0.05$, Fig 1). Expression of the short heterodimer partner (*Shp*) was similar in all three segments and significantly reduced in cholestatic and bile-deficient rats in the duodenum by ~60% and ~45% (both $P < 0.05$), in the jejunum by ~75% and ~70% (both $P < 0.01$) and in the ileum both by ~95% (both $P < 0.01$, Fig 1). Expression of *Gpbar1* was similar in duodenum, jejunum and ileum and was not affected by cholestasis or bile diversion (Fig 1).

After surgery the body weight decreased in all groups, but to a greater extent in cholestatic and bile-deficient rats compared to the sham-operated controls (93.7 ± 0.6 and 92.7 ± 0.7 vs. 97.5 ± 0.6% of initial weight at day 2 after surgery; both $P < 0.01$). Body weight of control rats remained stable over the experimental period, while body weight of bile-deficient rats

increased over time to the level of control rats at day 7. Body weight of the cholestatic rats remained stable over the experimental period and was significantly lower than body weight of control rats over the entire experimental period (approximately -4%, $P < 0.05$). Average food intake during the experimental period was similar in control and cholestatic rats (4.5 ± 0.2 and 4.1 ± 0.1 % of body weight). Average food intake of bile-deficient rats was significantly higher than that of cholestatic rats (4.9 ± 0.1 vs. 4.1 ± 0.1 % of body weight, $P < 0.01$) [10].

Fat balance was measured from day 5 to 6 after surgery (Fig 1). Fat ingestion was slightly, but not significantly higher in bile-deficient rats compared to control rats, as previously found [11]. Fecal fat excretion was significantly higher in cholestatic and bile-deficient rats compared to control rats (1.2 ± 0.1 and 1.2 ± 0.1 vs. 0.2 ± 0.0 mmol fatty acids/day, respectively, each $P < 0.01$). The resulting net fat absorption was significantly lower in cholestatic rats compared to control rats (2.9 ± 0.1 vs. 3.7 ± 0.2 mmol/day, respectively, $P < 0.05$), while net fat absorption in bile-deficient rats was not significantly different from the control rats. Cholestatic and bile-deficient rats had significantly lower coefficients of fat absorption than control rats (71.8 ± 0.5** and 73.1 ± 0.9** vs. 93.9 ± 0.2 % of ingested amount, respectively, each $P < 0.01$).

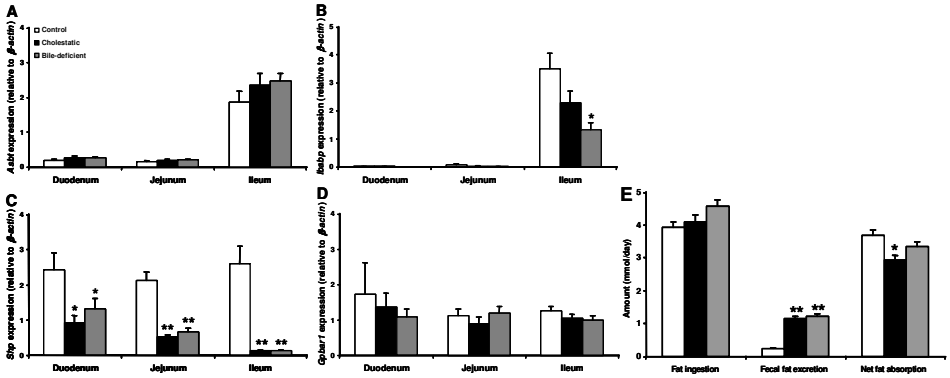

Figure 1. Gene expression of intestinal bile salt transporters/receptors and fat balance in control (white bars), cholestatic (black bars) and bile-deficient (grey bars) rats. (A) Duodenal, jejunal and ileal *Asbt* expression, (B) *Ibabp* expression, (C) *Shp* expression, and (D) *Gpbar1* expression, all normalized to *β-actin* levels. (E) Fat balance, measured from day 5 to 6 after surgery. Data represent means ± SE of 5-7 rats per group. *$P < 0.05$ and **$P < 0.01$ vs. control group.

Jejunal morphology, proliferation and apoptosis are not affected in cholestatic rats

Jejunal sections of control, cholestatic and bile-deficient rat intestines were stained with HE, Ki-67 and TUNEL to assess morphology, proliferation and apoptosis, respectively (Fig 2). Villous and crypt morphology, proliferation and apoptosis did not differ between the groups. Villous length was similar in control, cholestatic and bile-deficient rats (394 ± 36, 445 ± 26 and 510 ± 27 µm, respectively, Table 2), however, villous length in bile-deficient rats showed a trend towards being higher than villous length in control rats (Kruskal-Wallis H: $P = 0.064$ and Mann-Whitney U of bile-deficient rats vs. control rats: $P = 0.032$) [12]. Crypt/villous ratios were similar in control, cholestatic and bile-deficient rats (0.29 ± 0.02, 0.30 ± 0.02 and 0.28 ± 0.00, respectively, Table 2).

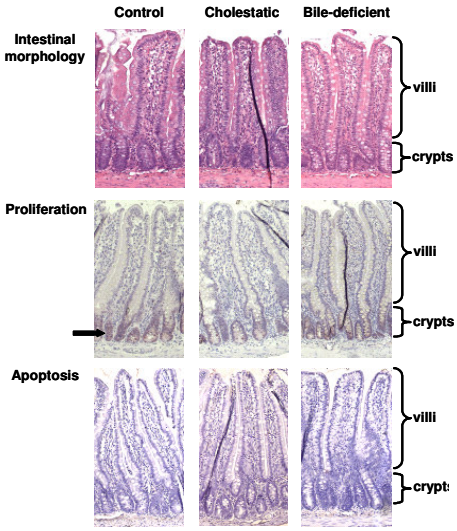

Table 2. Villus length and crypt-to-villus ratios of control, cholestatic and bile-deficient rats.

Group	Villus length (μm)	Crypt-to-villus length ratio
Control	394 ± 36	0.29 ± 0.02
Cholestatic	445 ± 26	0.30 ± 0.02
Bile-deficient	510 ± 27	0.28 ± 0.00

Figure 2. Jejunal morphology, proliferation and apoptosis in control, cholestatic and bile-deficient rats. Intestinal morphology was assessed by HE staining, proliferation by Ki-67 staining and apoptosis by TUNEL staining. Crypts and villi are indicated in the pictures. Cells positively stained with Ki-67 are indicated by the arrow.

Expression of intestinal transcription factors is maintained in cholestatic rats

The expression of intestine-specific transcription factors was determined to assess effects of the experimental manipulations on enterocyte differentiation. CDX-2, HNF-1α and GATA-4 are known to cooperatively regulate sucrase-isomaltase gene transcription [9]. *Hnf-1α* expression was similar in duodenum, jejunum and ileum, while *Gata-4* expression was significantly lower in the ileum compared to the duodenum and jejunum (both $P < 0.01$; Fig 3) [21] No significant differences in *Hnf-1α* or *Gata-4* expression were found among the groups. *Cdx-2* expression increased slightly from the proximal to the distal part of the intestine of control rats (NS; Fig 3). No significant differences were found in duodenum, jejunum and ileum among all groups. Cdx-2 protein expression was similar in all groups (Fig 3).

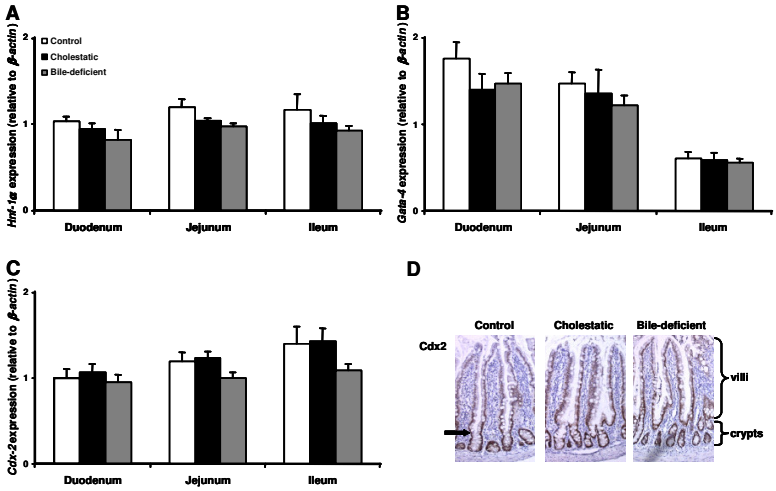

Figure 3. Expression of intestine-specific transcription factors in control (white bars), cholestatic (black bars) and bile-deficient (grey bars) rats. (A) Duodenal, jejunal and ileal expression of *Hnf-1α*, (B) *Gata-4*, and (C) *Cdx-2*, all normalized to *β-actin* levels. (D) Jejunal Cdx-2 protein expression. Cells positively stained with α-Cdx-2 are indicated by the arrow. Data represent means ± SE of 5-7 rats per group. *Gata-4* expression was higher in the duodenum and jejunum compared to the ileum ($P < 0.01$).

Blood glucose response is delayed in cholestatic rats

The blood glucose concentration in response to the intraduodenal bolus of labeled glucose and sucrose was delayed in cholestatic rats compared to bile-deficient and control rats and peaked at 15 min rather than at 7.5 min, respectively. Figure 4 shows significantly higher glucose concentrations in cholestatic rats compared to control and bile-deficient rats at 30 and 45 min after bolus administration. The area under the curve was not significantly changed in cholestatic rats compared to control and bile-deficient rats (1108 ± 51 vs. 1050 ± 40 and 1024 ± 28 mmol/L*min, respectively, NS).

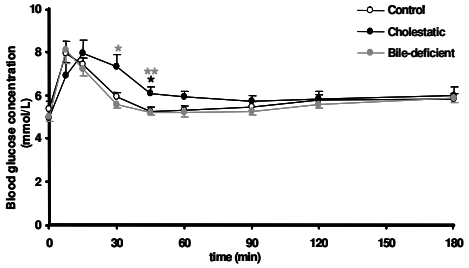

Figure 4. Blood glucose concentration in response to a 1 ml intraduodenal bolus containing ^{13}C-sucrose (0.25 g) and ^{2}H-glucose (1 mg) in control (white circles), cholestatic (black circles) and bile-deficient (grey circles) rats. Data represent means ± SE of 5-7 rats per group. *P < 0.05 vs. control group. $^{*}P$ < 0.05 and $^{**}P$ < 0.01 cholestatic vs. bile-deficient group.

Monomeric glucose absorption is maintained in cholestatic rats

Monomeric glucose absorption was assessed by determination of plasma appearance of ^{2}H-glucose after its intraduodenal administration. Plasma ^{2}H-glucose concentrations peaked at 7.5 min in control, cholestatic and bile-deficient rats (38 ± 4, 39 ± 6 and 41 ± 3 µmol/L, respectively; Fig 5). Areas under the curve were similar in cholestatic, control and bile-deficient rats (2220 ± 363, 1956 ± 315, and 1763 ± 222 µmol/L*min, respectively, NS).

In all groups, expression of the apical glucose transporter *Sglt-1* and that of the basolateral glucose transporter *Glut-2* were slightly lower in the ileum compared to the duodenum and jejunum (all $P < 0.05$; Fig 5). Jejunal *Sglt-1* expression was significantly increased in bile-deficient rats compared to control rats ($P < 0.01$). *Glut-2* expression was not significantly different between the three groups.

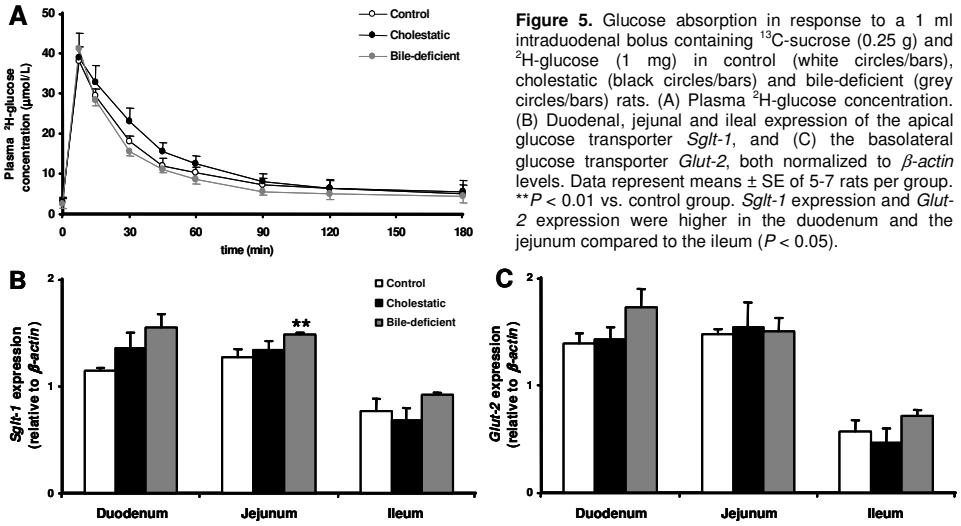

Figure 5. Glucose absorption in response to a 1 ml intraduodenal bolus containing ^{13}C-sucrose (0.25 g) and ^{2}H-glucose (1 mg) in control (white circles/bars), cholestatic (black circles/bars) and bile-deficient (grey circles/bars) rats. (A) Plasma ^{2}H-glucose concentration. (B) Duodenal, jejunal and ileal expression of the apical glucose transporter *Sglt-1*, and (C) the basolateral glucose transporter *Glut-2*, both normalized to *β-actin* levels. Data represent means ± SE of 5-7 rats per group. $^{**}P$ < 0.01 vs. control group. *Sglt-1* expression and *Glut-2* expression were higher in the duodenum and the jejunum compared to the ileum (P < 0.05).

Sucrose digestion is maintained in cholestatic rats

Sucrose digestion was assessed by appearance of plasma ^{13}C-glucose derived from ^{13}C-sucrose. Plasma ^{13}C-glucose concentrations peaked at 30 min in control, cholestatic and bile-deficient rats (3.4 ± 0.2, 4.1 ± 0.5 and 3.2 ± 0.3 mmol/L, respectively; Fig 6). Area under the curve was not significantly changed in cholestatic rats, when compared to control and bile-deficient rats (346 ± 42 vs. 312 ± 17 and 266 ± 21 mmol/L*min, respectively, NS). Sucrase enzyme activity was highest in the jejunum and lowest in the ileum in control rats (all $P < 0.01$; Fig 6). Duodenal sucrase enzyme activity was significantly lower in bile-deficient rats compared to control rats (6.9 ± 1.0 vs. 8.3 ± 0.8 μmol/mg protein.h, respectively, $P < 0.05$). The expression pattern of *sucrase-isomaltase (Si)* was less pronounced than that of sucrase enzyme activity, but jejunal expression was also significantly higher than ileal expression ($P < 0.05$). No differences were found among the groups.

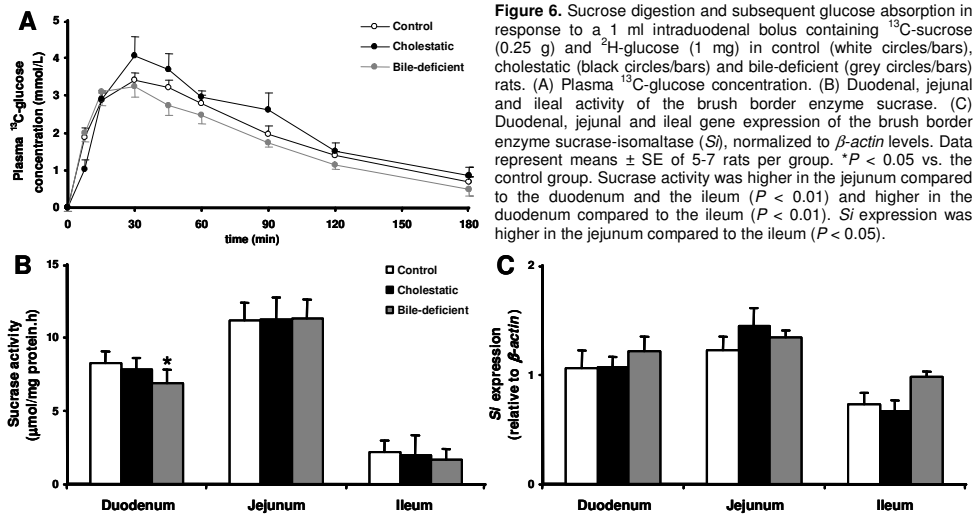

Figure 6. Sucrose digestion and subsequent glucose absorption in response to a 1 ml intraduodenal bolus containing ^{13}C-sucrose (0.25 g) and ^{2}H-glucose (1 mg) in control (white circles/bars), cholestatic (black circles/bars) and bile-deficient (grey circles/bars) rats. (A) Plasma ^{13}C-glucose concentration. (B) Duodenal, jejunal and ileal activity of the brush border enzyme sucrase. (C) Duodenal, jejunal and ileal gene expression of the brush border enzyme sucrase-isomaltase (*Si*), normalized to *β-actin* levels. Data represent means ± SE of 5-7 rats per group. *$P < 0.05$ vs. the control group. Sucrase activity was higher in the jejunum compared to the duodenum and the ileum ($P < 0.01$) and higher in the duodenum compared to the ileum ($P < 0.01$). *Si* expression was higher in the jejunum compared to the ileum ($P < 0.05$).

DISCUSSION

In this study, we investigated whether cholestasis; i.e. the combination of systemic accumulation of bile salts and the deficiency of bile salts in the intestinal lumen, affects the constitution and absorptive capacity of the rat small intestinal epithelium. We compared cholestatic rats with bile-deficient rats, without systemic accumulation, and with control rats. We found that short-term extrahepatic cholestasis in rats does not affect intestinal morphology, proliferation or apoptosis, nor the functional capacity of the intestine to digest sucrose and to absorb glucose.

Several studies describe the effect of the absence of bile components in the intestinal lumen on intestinal bile salt transporter expression in rats. They report that rat Asbt expression is not regulated by bile salts, while rat Ibabp expression is positively regulated by bile salts via Fxr activation [19,22-27]. In accordance with the literature, our data indicate that *Asbt* expression is not affected in cholestatic and bile-deficient rats. *Ibabp* expression was decreased in cholestatic rats and significantly decreased in bile-deficient rats. The increased expression of *Ibabp* in cholestatic rats compared to bile-deficient rats may be explained by the occurrence of retrograde transport of bile salts from the blood compartment into the

epithelial layer of the small intestinal lumen. However, expression of bile salt-sensitive *Shp* was markedly decreased in both cholestatic and bile-deficient rats. The strongest reduction was observed in the ileum, coinciding with the highest Fxr expression [28]. The bile duct-ligated rats had strongly elevated plasma bile salt levels and significantly reduced net fat absorption, in accordance with cholestasis. As previously described [10], cholestatic rats lost slightly more weight than bile-deficient and control rats, despite similar food intakes. The weight loss may be related to the decreased net fat absorption in cholestatic rats [13], compared with unchanged net fat absorption in bile-deficient rats fed a chow diet [11]. It is well-known that the composition of chow is variable between batches. Therefore we used only one batch to feed all rats before and during our experiments. Usage of a purified diet, such as AIN-93M, is not possible in this experimental setting, because the cornstarch in the diet is naturally enriched in ^{13}C as is the sucrose (cane sugar) we used to assess sucrose digestion. Pilot experiments showed that the enrichment of ^{13}C-glucose in the plasma is immeasurable, due to the high baseline enrichment in rats fed a diet containing cornstarch (unpublished observations).

In light of numerous *in vitro* data in the literature, we anticipated cholestasis to induce either proliferation or apoptosis in the small intestinal epithelium. Conjugated bile salts in concentrations found during cholestasis induce proliferation in the rat small intestinal cell line IEC-6, and in the human colon carcinoma cell line Caco-2, which gains small intestinal epithelial features upon differentiation [29-31]. In contrast to enterocytes, relatively low concentrations (50 µM) of conjugated bile salts induce apoptosis in human hepatoma cell lines and primary rat hepatocytes [32-34]. Bile salts in cholestatic concentrations can also be indirectly implicated in inhibition of differentiation and function of small intestinal epithelial cells. Suh and Traber demonstrated that the intestine-specific transcription factor Cdx-2 is an important regulator of differentiation in the small intestine [35]. Differentiation is regulated by phosphorylation and subsequent activation of Cdx-2 via the MAPK/ERK route. Phosphorylated Cdx2 inhibits transcription of the sucrase-isomaltase gene [36]. MAPK/ERK, in turn, appears to be activated by various bile salts [37]. However, our present results indicate that cholestatic concentrations of bile salts do not affect proliferation, apoptosis or differentiation in rat small intestinal epithelium *in vivo*. The discrepancy between the *in vitro* and *in vivo* data can possibly be explained by the fact that enterocytes might be more resistant in an *in vivo* setting, related to protective environmental factors. Besides, the absorption of nutrients and of bile salts takes place in different intestinal segments. In cholestatic rat models, enterocytes are exposed to bile salt concentrations only at their basolateral membrane, while intestinal cell lines are exposed to both sides or are not (completely) polarized. Bile salts do not need to enter the cells to activate cellular signaling routes. Kawamata *et al.* reported that membrane-type G protein coupled receptor Gpbar1 is expressed in the intestine [38]. In our rats, *Gpbar1* was expressed at similar amounts in duodenum, jejunum and ileum. It is unknown whether Gpbar1 is expressed at apical or basolateral membrane domains. Finally, several *in vitro* studies have addressed intestinal cell exposure to high concentrations of unconjugated bile salts in the context of colon cancer. Small intestinal cells *in vivo* are, however, normally not exposed to free bile salts in those concentrations and compositions.

Data addressing to what extent cholestasis or intestinal bile deficiency affect small intestinal function *in vivo* is scarce. In accordance with our current results several rat models of cholestasis or intestinal bile deficiency have been reported to have unchanged small intestinal function. Borges *et al.* reported that obstructive jaundice did not affect jejunal

absorption of glucose in rats [39]. Sucrase enzyme activity was shown to be unaffected in cholestyramine-fed and bile-diverted rats [40]. Corresponding with unaffected or minimally affected epithelial integrity, we have previously shown that fat absorption in 1 wk bile-diverted rats could be quantitatively reconstituted within hours using the infusion of model bile [41].

Vonk *et al.* developed a stable isotope test for the quantification of lactose digestion and glucose absorption in humans [15,16]. We adapted this test to quantify intestinal digestion and absorption of sucrose and glucose in rats. In the future, this test might be utilized or adapted to quantify the effect of other clinical conditions on intestinal absorption of carbohydrates or other macronutrients.

Malnutrition in children with cholestasis has a profound effect on mortality rate of children with end-stage cholestatic liver disease [5,7,42]. The association between cholestatic liver disease and fat malabsorption is well-known. Previous studies in our group have demonstrated that cholestatic rats and bile-deficient rats have severe fat malabsorption [11-13], as confirmed in the present study. Rings *et al.* showed that the absorption of free fatty acids but not fat digestion was rate-limiting for overall fat absorption in neonates that are known to have a mild 'physiological' cholestasis during the first months of life [43]. Our data indicate that intestinal deficiency of bile salts, with or without systemic accumulation of bile salts, does not impair intestinal carbohydrate digestion or absorption in rats. These observations suggest that increasing the dietary carbohydrate intake above conventional levels could be used in preventive or therapeutic fashion for a poor nutritional status induced by fat malabsorption in cholestatic children.

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CHAPTER 4

Cholestatic conditions and intestinal cell proliferation and differentiation

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ABSTRACT

Cholestasis in children impairs their nutritional status and growth. Recently we demonstrated in a rat model of cholestasis that increased plasma bile salt concentrations did not affect intestinal carbohydrate digestion or absorption. We addressed to what extent cholestatic conditions affect proliferation or differentiation of enterocytes *in vitro*, and what underlies their apparent resistance to bile salts. We studied proliferation and differentiation of an intestinal cell line, Caco-2, exposed to bile salts in cholestatic concentrations. Exposure of proliferating or short-term differentiated cells to cholestatic conditions did not affect DNA content, cell number or sucrase activity. In contrast, exposure of long-term differentiated cells to cholestatic conditions reduced sucrase activity, coinciding with increased expression of bile salt transporter ASBT. Unconjugated CDCA reduced sucrase activity earlier in the differentiative phase. Present data suggest that enterocytes are protected from bile salt-induced effects through absence of bile salt transporter ASBT. These data were confirmed *in vivo*.

INTRODUCTION

Cholestatic liver disease negatively affects nutritional status and growth in children. For cholestatic children requiring liver transplantation, a poor nutritional status imposes an increased risk of morbidity and mortality. Malnutrition and growth retardation in cholestatic disorders are usually ascribed to insufficient fat solubilization and absorption due to reduced presence of bile components in the intestinal lumen [1-3].

Cholestasis is also characterized by elevated bile salt plasma concentrations, which theoretically could affect intestinal function. Lower concentrations of bile salts have been shown to induce proliferation, differentiation or apoptosis in intestinal epithelial cell lines, depending on hydrophobicity and cell line [4-6]. In contrast to *in vitro* data, we recently demonstrated that carbohydrate digestion and absorption was maintained in a rat model of cholestasis, but we could only speculate about the mechanism underlying the functional protection against cholestatic conditions [7].

To address this issue, we exposed the human colon carcinoma cell line Caco-2 to cholestatic concentrations of conjugated bile salts and assessed bile salt transport parameters, proliferation, differentiation and apoptosis. Caco-2 cells develop small intestinal features upon reaching confluency [8]. We separately studied proliferating Caco-2 cells, which can be regarded as a model for proliferating crypt cells, and differentiated Caco-2 cells, which can be regarded as a model for differentiated villous cells of the small intestine. Results were compared to those of unconjugated chenodeoxycholic acid (CDCA) since it can enter the cells independent of transporters as opposed to conjugated bile salts.

Finally, we studied at the interaction between nutrient absorption and bile salt absorption *in vivo* by looking at the correlation between expression of proteins involved in carbohydrate digestion and absorption in the ileal part of the small intestine and plasma bile salt concentrations in cholestatic, bile-deficient and control rats.

Present data indicate that exposure of proliferating and short-term differentiated cells to conjugated bile salts in cholestatic concentrations did not affect Caco-2 cell proliferation or differentiation. Exposure of long-term differentiated cells to cholestatic conditions reduced differentiation, coinciding with increased expression of bile salt transporter ASBT.

MATERIALS AND METHODS

Materials

The bile salts glycocholic acid (GCA) and CDCA, (sodium salts), were from Calbiochem (San Diego, CA, USA). Taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA), (sodium salts), were from Sigma (St. Louis, MI, USA). All bile salts were dissolved in demineralized H_2O in 50 mM stock solutions and mixed with the medium before addition to the cells.

Cell culture

The human colon carcinoma cell line Caco-2 was obtained from the American Type Tissue Culture Collection (Manassas, VA, USA) and maintained in DMEM (Gibco BRL, USA) supplemented with 10% (vol/vol) FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids and 0.25% human transferrin in a humidified atmosphere of 95% air–5% CO_2 at 37°C. For the experiments, cells were used between passage 15 and 30.

Experimental design

Caco-2 cells were seeded at $5x10^4$ cells per well in 6-well plates (Corning Costar, Cambridge, MA, USA). Caco-2 cells were exposed to 0, 150, 300 and 450 µmol/L of a bile salt mixture containing GCA, TCA, GCDCA and TCDCA in a 2:2:1:1 molar ratio or to 250 µmol/L CDCA. The effects of bile salts on proliferating and differentiated Caco-2 cells were assessed by exposing the cells to bile salts 24h after seeding and after confluency was reached (~8 days), respectively. Proliferating cells were harvested at day 0, 2, 4, 6 and 14 (P0, P2, P4, P6 and P14) and differentiated cells were harvested at day 0, 7, 14 and 21 (D0, D7, D14 and D21). The effect of exposure to CDCA was assessed at P4, P14, D7, D14 and D21. Caco-2 cells in Transwell plates were harvested at day 7, 14 and 21 (D7, D14 and D21). Medium with or without bile salts was changed every 2-3 days.

Analyses

Cell numbers were counted manually with the Burker Cell System. DNA was quantified with a DNA quantification kit (Sigma, St. Louis, MI, USA) according to the manufacturer's instructions. Caspase-3 activity was measured with the CaspACE™ Assay System (Promega, Madison, USA) according to the manufacturer's instructions. mRNA expression levels were measured by real-time PCR, as described previously [9]. PCR results were normalized to GAPDH mRNA levels. The sequences of the primers and probes are listed in Table 1. Enzyme activity levels of sucrase were measured as described by Dahlqvist [10]. Activity levels were normalized to protein levels, measured by the BCA method as described by the manufacturer (Pierce, Rockford, IL, USA). Lactate dehydrogenase (LDH) in the medium was quantified as described by Smit *et al.* [11].

Table 1. Primer and probe sequences

Gene	GenBank	Forward Primer	Reversed Primer	TaqMan® probe
GAPDH	NM_002046	GGT GGT CTC CTC TGA CTT CAA CA	GTG GTC GTT GAG GGC AAT G	ACA CCC ACT CCT CCA CCT TTG ACG C
FXR	AF384555 (α1) NM_005123 (α2)	TTC TGA AAA TTT ATT TGG TGT TTT AAC AGA	TGG AAT AAT AGG ATG ACG AGG AAA TC	CAT TGC TGT ATT GCG AGT ATG GTT CCA CTT CC
ASBT	NM_000452	CAC GCA GCT ATG TTC CAC CAT	GAG CGG AAG GTG AAT ACG A	CAG CTC TCC TTC ACT CCT GAG GAG CTC A
IBABP	NM_001445	AAG GCC CGC AAC TTC AAG AT	GGA GTA GTG CTG GGA CCA AGT G	CCA TCC TGC TGC ACC TCC GTG A
GPBAR1	NM_001077191.1	CGT CTA CTT GGC TCC CAA CTT C	GGC CTC AGG ACT GCC ATG TA	CTC TCC CTG CTT GCC AAC CTC TTG C
SI	NM_001041	TGT GGA TAA ATG AGT CAG ATG GAA G	GCC CAC CAA TCA ATG CAG TT	ACT GTT AAT CCT GGC CAT ACC TCT CCA ATA A
CDX-2	NM_001265	CTC GGC AGC CAA GTG AAA AC	TGG TGA TGT AGC GAC TGT AGT GAA	CCT TCT CCA GCT CCA GCC GCT G
GATA-4	NM_002052.2	GGA GAT GCG TCC CAT CAA GA	CTG ACT GAG AAC GTC TGG GAC A	CAT CTC ACT ACG GGC ACA GCA GCT CC

Gene expression and plasma bile salt concentrations in cholestatic, bile-deficient and control rats

mRNA levels of SI, Sglt-1 and Glut-2 and plasma bile salt concentrations in cholestatic, control and bile-deficient rats were obtained and statistically tested as described previously [7].

Statistical analysis

Values represent means ± SEM of 3-6 experiments. Using SPSS version 12.0.2 statistical software (Chicago, IL, USA), we calculated significance of differences with the Mann-

Whitney *U*-test. Statistical information is provided regarding differentiative (D0) vs. proliferative cells (P0), alterations during the differentiative phase (D0 vs. D21) and control cells vs. conjugated bile salts in cholestatic concentrations (450 μmol/L) or CDCA (250 μmol/L). $P<0.05$ was considered significant.

RESULTS

Cholestatic conditions increase IBABP expression in proliferating and differentiated cells.

First, we assessed key parameters regarding to bile salt transport and signaling; bile salt-activated farnesoid X receptor (FXR), apical sodium-dependent bile salt transporter (ASBT), ileal bile acid-binding protein (IBABP) and G protein-coupled membrane bile acid receptor (GPBAR).

In Figure 1 is shown that the expression of *FXR* was increased (~5-fold; $P<0.05$) in differentiated cells (D0) compared to proliferating cells (P0). *FXR* expression was further increased during the differentiative phase (D0 vs. D21) of Caco-2 cells (~11-fold; $P<0.01$). Similarly, *ASBT* expression was increased in differentiative cells (~3-fold; $P<0.05$) and during the differentiative phase (~7-fold; $P<0.01$). In contrast, *IBABP* expression and *GPBAR1* expression (data not shown) were not induced during the differentiative phase, but were increased in differentiative cells compared to proliferative cells (~4-fold; $P<0.01$ and ~1.5-fold; $P<0.05$, respectively; Fig 1).

Exposure to conjugated bile salts in cholestatic concentrations significantly increased *IBABP* expression at day 6 (~2-fold; $P<0.01$) and 14 (~20-fold; $P<0.05$) in proliferating cells and at day 7-21 (~20-35 fold; $P<0.01$) in differentiated cells, while exposure to conjugated bile salts in cholestatic concentrations did not affect *FXR*, *ASBT* or *GPBAR1* expression.

In contrast to conjugated bile salts in cholestatic concentrations, CDCA significantly reduced *ASBT* expression in differentiated cells (by ~40% at day 14 and by ~75% at day 21; both $P<0.05$, Fig 1). *IBABP* expression was strongly induced upon exposure to CDCA in differentiated cells (Fig 1).

Conjugated bile salts in concentrations of 150 and 300 μmol/L generated similar results as 450 μmol/L. For sake of clarity, we only present results of cells exposed to 450 μmol/L conjugated bile salts or 250 μmol/L CDCA compared to control cells.

Cholestatic conditions do not affect DNA content or cell number in proliferating or differentiated cells.

DNA content and cell number were quantified to assess cell proliferation under cholestatic conditions. DNA content in differentiative cells was ~9-fold ($P<0.01$) higher compared to proliferative cells, independent of the presence or absence of conjugated bile salts. During the differentiative phase, however, DNA content remained similar. CDCA decreased DNA content in proliferating cells, but not in differentiated cells (Fig 2).

Cell number showed a similar pattern during differentiation. Cholestatic bile salt concentrations did not affect cell number at any time point, but CDCA reduced cell number in proliferating cells (-~ 99%, $P<0.01$, data not shown).

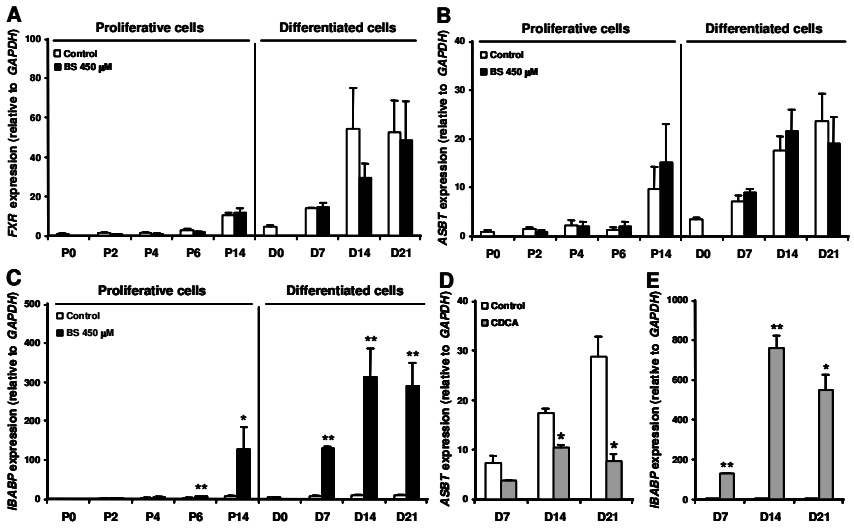

Figure 1. Bile salt transporter/receptor gene expression in Caco-2 cells exposed to conjugated bile salts in cholestatic concentrations (450 µmol/L) or CDCA (250 µmol/L) compared to control cells. (A) FXR, (B) *ASBT*, (C) *IBABP* expression, normalized to *GAPDH*, in cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. (D) *ASBT*, (E) *IBABP* expression, normalized to *GAPDH*, in cells exposed to CDCA compared to control cells. P0, P2, P4, P6 and P14 represent proliferating cells at day 0, 2, 4, 6 and 14 after exposure to bile salts and D0, D7, D14 and D21 represent differentiated cells at day 0, 7, 14 and 21 after exposure to bile salts. **P*<0.05 and ***P*<0.01.

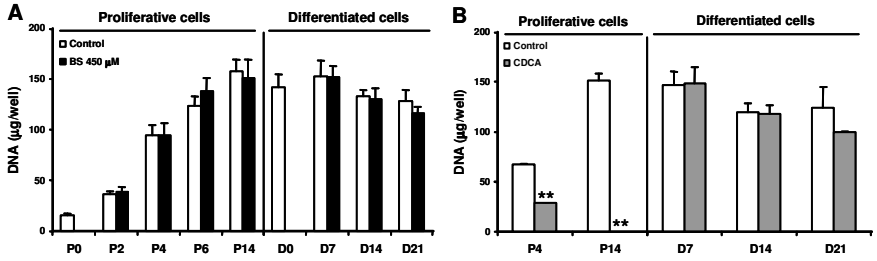

Figure 2. DNA content of Caco-2 cells exposed to conjugated bile salts in cholestatic concentrations (450 µmol/L) or CDCA (250 µmol/L) compared to control cells. (A) DNA content of cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. (B) DNA content of cells exposed to CDCA compared to control cells. P0, P2, P4, P6 and P14 represent proliferating cells at day 0, 2, 4, 6 and 14 after exposure to bile salts and D0, D7, D14 and D21 represent differentiated cells at day 0, 7, 14 and 21 after exposure to bile salts. ***P*<0.01.

Cholestatic conditions do not affect caspase-3 activity in proliferating or differentiated cells.

Caspase-3 activity was quantified to assess the occurrence of apoptosis. Caspase-3 activity was very low and similar in control cells and in cells exposed to conjugated bile salts in cholestatic concentrations. Unconjugated CDCA increased caspase-3 activity by ~50% in proliferating cells (*P*<0.05, data not shown).

LDH leakage in the medium was quantified to assess potential bile salt-induced cytotoxicity. Neither in proliferating nor differentiated cells, LDH leakage was induced by exposure to conjugated bile salts in cholestatic concentrations or unconjugated CDCA (data not shown).

Cholestatic conditions reduce sucrase enzyme activity in long-term differentiated cells.

Sucrase enzyme activity and sucrase-isomaltase (SI) mRNA levels were quantified as markers for Caco-2 cell differentiation. Sucrase enzyme activity was markedly induced (~27-fold; $P<0.05$) in differentiative cells compared to proliferative cells and further during the differentiative phase (~16-fold; $P<0.01$) as also described by Van Beers *et al.* [12]. Exposure to conjugated bile salts in cholestatic concentrations did not affect sucrase enzyme activity in proliferating cells or in differentiated cells up to day 7. Conjugated bile salts significantly reduced sucrase enzyme activity in differentiated cells after day 14 ~20-25%; $P<0.05$; Fig 3). Unconjugated CDCA abolished sucrase enzyme activity in proliferating cells at day 14, and in differentiated cells from day 14 on (by ~25-45%; $P<0.01$; Fig 3).

The expression pattern of SI mRNA during differentiation was comparable to that of sucrase enzyme activity. *SI* expression was strongly increased in differentiative cells compared to proliferative cells (~81-fold; $P<0.01$) and further increased during the differentiative phase (~5-fold; $P<0.01$). Conjugated bile salts in cholestatic concentrations significantly reduced *SI* expression in proliferating cells at day 2 (by ~65%; $P<0.05$). In differentiative cells, *SI* expression was also reduced upon exposure to conjugated bile salts, but this did not reach statistical significance (Fig 3).

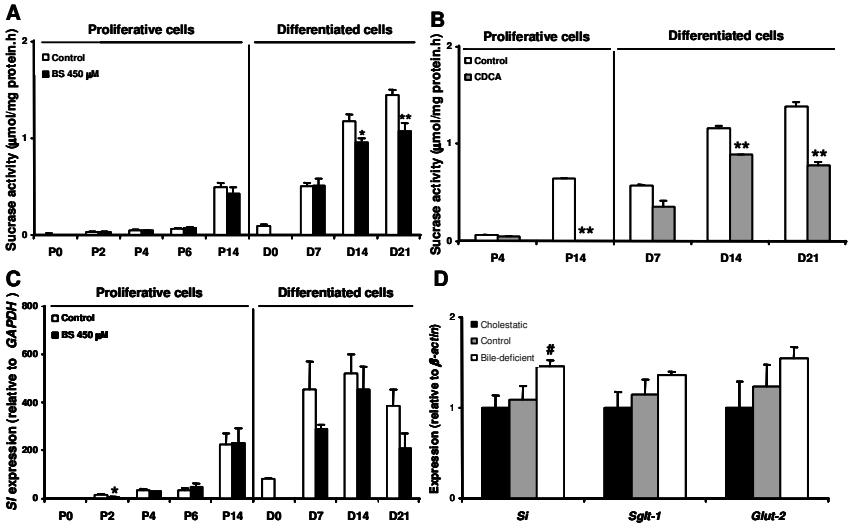

Figure 3. Sucrase enzyme activity and sucrase-isomaltase (SI) expression in Caco-2 cells exposed to conjugated bile salts in cholestatic concentrations (450 µmol/L) or CDCA (250 µmol/L) compared to control cells. (A) Sucrase enzyme activity in cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. (B) Sucrase enzyme activity in cells exposed to CDCA compared to control cells. (C) *SI* expression, normalized to GAPDH, in cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. P0, P2, P4, P6 and P14 represent proliferating cells at day 0, 2, 4, 6 and 14 after exposure to bile salts and D0, D7, D14 and D21 represent differentiated cells at day 0, 7, 14 and 21 after exposure to bile salts. *$P<0.05$ and **$P<0.01$. (D) Ileal expression of *SI*, *Sglt-1* and *Glut-2* in cholestatic (black bars), control (grey bars) and bile-deficient (white bars) rats, normalized to β-actin. Values of cholestatic rats are set on 1. Plasma bile salt concentrations cover a range from 187±20 µmol/L in cholestatic rats, 10±4** µmol/L in control rats to 1.0±0.5**$^{\#\#}$ µmol/L in bile-deficient rats of which **$P<0.01$ vs. cholestatic group and $^{\#\#}P<0.01$ vs. control group. $^{\#}P=0.064$ (Kruskal-Wallis H) and $P=0.032$ (Mann-Whitney U) vs. control group.

Plasma bile salt concentrations correlate inversely with ileal SI, Sglt-1 and Glut-2 expression in rats.

As stated before, we previously demonstrated that intestinal carbohydrate absorption was maintained in cholestatic rats [7]. Figure 3D shows mRNA levels of SI, and intestinal glucose transporters Sglt-1 (apical) and Glut-2 (basolateral) in the ileal segment of the small intestine of cholestatic, control and bile-deficient rats. The average plasma bile salt concentrations in cholestatic, control and bile-deficient rats were 187±20, 10±4 (P<0.01 vs. cholestatic rats) and 1.0±0.5 (P<0.01 vs. cholestatic and control rats) µmol/L, respectively. Though not significantly different among the groups, a clear pattern can be observed. *SI*, *Sglt-1* and *Glut-2* expression showed a trend towards being higher in bile-deficient rats compared to control and cholestatic rats. *SI* expression was increased by ~45% in bile-deficient rats compared to cholestatic rats (Kruskal-Wallis H: P=0.064 and Mann-Whitney U: P=0.028; Fig 3).

Cholestatic conditions do not affect CDX-2 or GATA-expression in proliferating and long-term differentiated cells.

CDX-2 and GATA-4 are intestine-specific transcription factors known to cooperatively regulate sucrase-isomaltase gene transcription [13]. Expression of *CDX-2* and *GATA-4* was, respectively, ~3-fold (P<0.05) and ~5-fold (P<0.01) higher in differentiative cells compared to proliferative cells. During the differentiative phase, CDX-2 and GATA-4 expression showed similar patterns, specifically slight inductions of expression at day 7 and 14, returning to base level at day 21 (Fig 4).

CDX-2 expression was not changed upon exposure to conjugated bile salts. Conjugated bile salts in cholestatic concentrations did not affect *GATA-4* expression in proliferating or differentiated cells, except for day 7 in differentiated cells (~-35%; P<0.05, Fig 4).

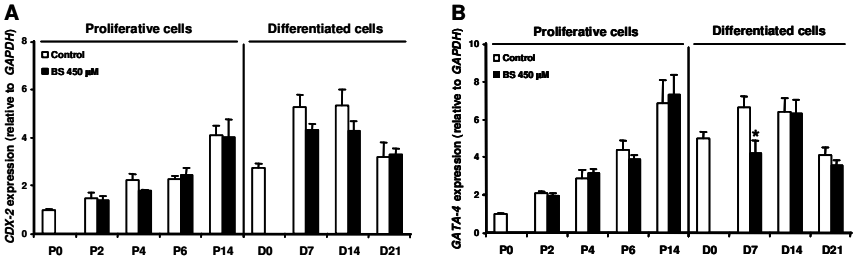

Figure 4. Gene expression of intestine-specific transcription factors in Caco-2 cells exposed to conjugated bile salts in cholestatic concentrations (450 µmol/L) compared to control cells. (A) *CDX-2*, (B) *GATA-4* expression, both normalized to *GAPDH*, in cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. P0, P2, P4, P6 and P14 represent proliferating cells at day 0, 2, 4, 6 and 14 after exposure to bile salts and D0, D7, D14 and D21 represent differentiated cells at day 0, 7, 14 and 21 after exposure to bile salts. *P<0.05.

DISCUSSION

We recently demonstrated that carbohydrate digestion and absorption is maintained in a rat model of cholestasis [7]. In this study we set out to find an explanation for enterocyte protection against cholestatic conditions. We found that exposure of proliferating and short-term differentiated Caco-2 cells to conjugated bile salts in cholestatic concentrations did not affect proliferation or differentiation, whereas exposure of long-term differentiated cells to cholestatic conditions reduced differentiation, coinciding with increased expression of bile salt transporters.

The lifespan of an enterocyte between crypt cells entering the villous-crypt junction, migrating upwards reaching the villous tip and eventually shedding off, ranges from approximately 2 to 3 days in mice and rats to 6 days in humans [14,15]. Based on this physiological knowledge, one would consider short-term exposure as physiologically most relevant. We realized, however, that a cell model may not mimic physiological conditions in this respect. We first determined relevant features of Caco-2 cells at different developmental stages, including long-term differentiated cells, to find the condition most relevant to study our research question.

Our results clearly showed a discrepancy with respect to proliferating–short-term differentiated cells and long-term differentiated cells, i.e. exposure to cholestatic conditions in long-term differentiated cells reduced differentiation in contrast to proliferating and short-term differentiated cells. This discrepancy might be due to the higher quantity of the bile salt transporters in long-term differentiated cells compared to proliferating and short-term differentiated cells. Corresponding with this, our data indicate that ASBT mRNA levels are strongly increased in long-term differentiated cells compared with proliferative and short-term differentiative cells, coinciding with increased FXR mRNA levels as previously described by De Gottardi *et al.* [16]. Though bile salts seem to be able to enter cells with relatively low *ASBT* expression, indicated by reduced *IBABP* expression upon exposure to conjugated bile salts in proliferating and short-term differentiated cells, the quantity is most likely to low to exert effects on sucrase activity. Unconjugated CDCA can cross the cellular membrane independent of active transporters. The observation that exposure to CDCA already reduces sucrase activity earlier in the differentiative phase is compatible with the theory stated above.

Bile salts, however, do not need to enter the cells to activate cellular signaling. Marayuma *et al.* identified a cell surface G protein-coupled receptor, GPBAR1, responsive to low concentrations of extracellular bile salts [17]. Human GPBAR1 mRNA has been shown to be expressed in the small intestine and activation by bile salts leads to the activation of cellular signaling routes [18]. The elevated plasma bile salt level during cholestasis could theoretically activate signaling routes via GPBAR1. Since GPBAR1 was present in proliferating and differentiated Caco-2 cells, cholestatic bile salt concentrations might be able to influence enterocyte proliferation or differentiation via GPBAR1. The observation that reduced activity and mRNA levels of sucrase did not coincide with changes in GPBAR1 expression, however, does not support this theory.

Effects of bile salts on human intestinal cell proliferation range from inhibition to induction, usually related to the hydrophobicity of the bile salt. Unconjugated CDCA induces profound apoptosis in human intestinal cells, while similar concentrations of conjugated bile salts induce proliferation [5]. Effects of bile salts on intestinal cell differentiation have not been studied extensively. Ursodeoxycholic acid (UDCA) has been found to induce differentiation in a HCT116 cell line, derived from human colon carcinoma cells [4]. Interestingly, in culture,

hepatocytes seem to be much more sensitive to bile salt exposure than enterocytes. Glycochenodeoxycholic acid already induces apoptosis at 50 µmol/L in hepatocytes, while similar concentrations do not affect enterocytes *in vitro* [6]. We cannot appoint a cause for the differences in behavior between hepatocytes and enterocytes exposed to bile salts, but we can speculate about the underlying basis. First, hepatocytes possibly possess more bile salt uptake transporters than enterocytes, with possible higher affinity. Second, cell signaling pathways might be differentially regulated.

Nutrient absorption is most efficient in the jejunal part of the small intestine, while active bile salt absorption is restricted to the terminal ileum [19]. This observation would suggest that enterocytes involved in nutrient absorption are protected from bile salts. The observation that reduced sucrase enzyme activity in long-term differentiated cells exposed to conjugated bile salts in cholestatic concentrations coincided with high expression of the bile salt transporter ASBT, supports this theory. Thus, bile salts could potentially be harmful for enterocyte function when nutrient absorption and bile salt absorption would have been localized in the same intestinal segment. The significance of different intestinal segments involved in nutrients absorption and bile salt reabsorption has been well defined by Bosse *et al* [20]. They identified Gata-4 as an essential mediator of the maintainance of jejunal-ileal identities in mice. Synthesis of a transcriptionally inactive Gata-4 mutant in the mouse jejunum resulted in an attenuation of expression of genes involved in nutrient absorption and an induction of genes involved in bile salt absorption [20]. Moreover, results from our *in vivo* study showed reduced sucrase activity in rats with higher plasma bile salt concencentrations (bile-deficient > control > cholestatic rats) in the ileal part of the small intestine, while this pattern was not observed in the duodenal and jejunal part of the intestine [7]. The effect could possibly be more pronounced in the terminal ileum of rats exposed to even higher plasma bile salt concentrations, as seen in human cholestasis.

Since Caco-2 cells are derived from colon carcinoma cells it is impossible to ascribe features gained upon differentiation to any part of the small intestine. However, since differentiated Caco-2 cells express proteins involved in nutrient absorption as well as proteins involved in bile salt absorption, it is the ideal model to study interactions between these processes.

In conclusion, our data indicate that intestinal epithelial cells involved in nutrient absorption are resistant to exposure to conjugated bile salts in cholestatic concentration and composition. The maintenance of intestinal cell proliferation and differentiation under cholestatic conditions suggests that the absorptive function of the small intestine is preserved, which is most likely related to the fact that nutrient absorption and bile salt absorption are localized in different parts of the intestine.

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CHAPTER 5

Essential fatty acid deficiency in mice impairs lactose digestion

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Submitted

ABSTRACT

Background: Essential fatty acid (EFA) deficiency in mice induces fat malabsorption. We previously reported indications that the underlying mechanism is located at the level of the intestinal mucosa.

Aim: To characterize the effects of EFA deficiency on small intestinal morphology and function.

Methods: Mice were fed an EFA-deficient or control diet for 8 weeks. A 72 h fat balance, the EFA status, and small intestinal histology were determined. Carbohydrate absorptive and digestive capacities were assessed by stable isotope methodology after administration of U-^{13}C-glucose and 1-^{13}C-lactose. Concentrations of the EFA linoleic acid (LA), and the enzyme activity and mRNA expression of lactase, were measured in small intestinal mucosa.

Results: Mice fed the EFA-deficient diet were markedly EFA-deficient with a profound fat malabsorption. EFA deficiency did not affect the histology or proliferative capacity of the small intestine. Blood $^{13}C_6$-glucose appearance and disappearance were similar in both groups, indicating unaffected monosaccharide absorption. In contrast, blood appearance of ^{13}C-glucose, originating from ^{13}C-lactose, was delayed in EFA-deficient mice. EFA deficiency profoundly reduced lactase activity (-58%, $p<0.01$) and mRNA expression (-55%, $p<0.01$) in mid small intestine. Both lactase activity and its mRNA expression strongly correlated with mucosal LA concentrations ($r=0.89$ and 0.79, resp., $p< 0.01$).

Conclusions: EFA deficiency in mice inhibits the capacity to digest lactose, but does not affect small intestinal histology. These data underscore the observation that EFA deficiency functionally impairs the small intestine, possibly mediated by low LA levels in the enterocytes.

INTRODUCTION

Essential fatty acid (EFA) deficiency can occur in cholestatic liver diseases as a consequence of fat malabsorption [1,2]. However, EFA deficiency itself also induces fat malabsorption [3,4]. The underlying mechanism of EFA-deficient fat malabsorption remains unclear. Absorption of fat involves lipolysis, solubilization and intestinal translocation from the lumen into the mucosa, chylomicron assembly and transport into the lymph [5,6]. Previous studies in EFA-deficient mice have indicated that impaired lipolysis or bile formation do not cause the fat malabsorption in EFA deficiency [4]. Recently we reported data to suggest that EFA deficiency in mice affects fat absorption at the level of the small intestinal mucosa [4]. However, it has not been proven that EFA deficiency impairs the mucosal phase of fat absorption.

In contrast to fat absorption, the absorption of di- and monosaccharide carbohydrates exclusively depends on mucosal function [7]. The monosaccharide glucose is actively transported across the brush border membrane in the small intestine by the brush-border transporter SGLT1 (Sodium-dependent glucose transporter) [7,8]. The disaccharide lactose is first hydrolyzed by the mucosal membrane anchored lactase-phlorizin hydrolase (lactase, LPH) into glucose and galactose, prior to their active transport across the brush border by SGLT1 [8,9]. Besides being an important enzyme in lactose hydrolysis, lactase is a marker of enterocyte differentiation [10]. Throughout development total intestinal lactase activity remains similar to that found in newborns [11]. This characteristic makes lactase a good marker for functional assessment of the small intestine in adult animal.

Essential fatty acids (EFAs) are structural components of membrane phospholipids. Enterocyte membrane phospholipids are particulary rich in linoleic acid (LA, C18:2n-6) [12] which is necessary for modulations of a wide variety of biological functions and for physiochemical adaptations of the membrane lipid matrix to alterations in membrane fluidity [13]. The lipid matrix influences the conformation and function of proteins embedded in the inner and/or outer leaflet of the membrane [14]. Recently, an additional role of EFAs in alterations of bilayer elastic properties and lipid composition in lipid rafts have been reported [15,16]. Through activation of peroxisome proliferator-activated receptors (PPARs), EFAs can regulate transcriptional activity of several genes, including of those involved in fatty acid transport and metabolism [17,18].

In the present study we characterized the effects of EFA deficiency on small intestinal morphology and function in mice. Korotkova *et al.* have shown that EFA deficiency affects the fatty acid composition in the phospholipids of the rat small intestinal mucosa by decreasing the jejunal concentrations of linoleic acid [12]. However, no studies have been performed on the effect of EFA deficiency on the small intestinal function. We assessed the absorption of glucose, a major source of metabolic energy for mammalian cells [19], and lactose, as appropriate functional markers of the small intestine, in a previously developed and characterized murine model of EFA deficiency [4]. We applied stable isotope methodology [20], since this approach allows extension to similar studies in patients with EFA deficiency, cholestasis, or other forms of malabsorption [21-23]. U-^{13}C-labeled glucose and 1-^{13}C-labeled lactose were administered to EFA-deficient and control mice. Blood appearance of labels derived from administered glucose ($^{13}C_6$-glucose) and lactose (^{13}C-glucose) into the blood glucose fraction was subsequently quantified. We also determined the activity and expression of lactase, as well as the concentration of LA, in the mucosa along the proximal-to-distal axis of the small intestine.

Our data show that EFA deficiency is associated with impaired lactose digestion in mice. This functional observation corresponds with lower lactase mRNA expression and enzyme activity in the mid small intestine of EFA-deficient mice, accompanied by low LA concentrations.

MATERIALS AND METHODS

Mice and housing

Wild type mice with a free virus breed (FVB) background were obtained from Harlan (Horst, The Netherlands). Male mice (25-35 g) were housed in a light-controlled (lights on 6 AM-6 PM) and temperature-controlled facility and were allowed tap water and chow (AB diets, Woerden, Netherlands) *ad libitum*. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, the Netherlands.

Materials

U-^{13}C-glucose and 1-^{13}C-lactose were obtained from Isotec Inc. (Miamisburg, Ohio, USA) with isotopic enrichments of 99%. Unlabeled lactose was obtained from Fluka (Buchs, Switzerland).

Experimental diets

Similar to previous studies, we used high-fat EFA-deficient and EFA-sufficient (control) diets (16 wt% and 34 energy% fat), in order to mimic more closely the human diet composition [4]. The diets were custom synthesized by Arie Bloks BV (Woerden, the Netherlands, diet codes EFA deficient #4141.08 and EFA-sufficient #4141.07). The EFA-deficient diet contained 64 mol% palmitic acid (C16:0), 18 mol% stearic acid (C18:0), 13 mol% oleic acid (C18:1n-9) and 5 mol% linoleic acid (C18:2n-6). The isocaloric EFA-sufficient diet contained 36 mol% C16:0, 5 mol% C18:0, 31 mol% C18:1n-9 and 29 mol% C18:2n-6. Fatty acid contents of the diets were analyzed by extracting, hydrolyzing and methylating total dietary fatty acids as described by Muskiet *et al.* and subsequent separation and quantification of fatty acid methyl esters was performed by gas chromatograhy as described previously [4,24].

Experimental procedures

Induction of EFA deficiency. Mice were fed standard laboratory chow containing 6 weight% fat from weaning, and were switched to EFA-deficient or control diet at eight weeks of age. At the end of eight weeks-period on EFA-deficient or control diet, fat absorption was assessed by measuring food intake and collecting feces for 72 h. Following this eight weeks-period the mice underwent a glucose/lactose absorption test with U-^{13}C- glucose and 1-^{13}C-lactose (details see below) [24,25]. After the test the mice were anesthetized and sacrificed by obtaining a large blood sample through cardiac puncture for determination of erythrocyte EFA-status by the triene/tetraene (C20:3n-9/C20:4n-6) ratio [4]. The small intestine was excised, flushed with ice-cold PBS and divided into a proximal, mid and distal segment of similar size. Smaller parts from the middle of each small intestinal segment were harvested for histology and gene expression. The remaining part of the small intestine was opened lengthwise and the mucosa was removed by scrapping the luminal surface with a glass

coverslip. Mucosa was homogenized in buffer (see below for details) and used for the determination of enzyme activity, proteins and LA concentrations in mucosal phospholipids.
Glucose/lactose absorption. Glucose absorption and lactose digestion were determined by a combined U-^{13}C-glucose/1-^{13}C-lactose absorption test. After an overnight fast, mice received 0.5 mg U-^{13}C-glucose, 5 mg 1-^{13}C-lactose and 5 mg naturally enriched lactose in 300 µL PBS via gastric gavage. Before and at time points 7.5, 15, 30, 45, 60, 90, 120 and 180 min. after administration, blood samples were obtained by blood spot technique from the tail for determination of blood concentrations of (total) glucose, $^{13}C_6$-glucose (glucose originating from U-^{13}C-glucose) and ^{13}C-glucose (originating from 1-^{13}C-lactose) [25]. For reasons of clarity, we will address "blood" $^{13}C_6$-glucose and ^{13}C-glucose as "plasma" in the Results and Discussion sections.

Analytical methods
Lipid absorption, triene/tetraene ratio, blood glucose and serum insulin concentrations. Lipid absorption and erythrocyte triene/tetraene ratio were determined as described previously [4,27]. Blood glucose levels were measured with a Lifescan EuroFlash glucose meter (Lifescan Benelux, Beerse, Belgium). Insulin was measured in a solid phase two-site enzyme immunoassay in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule (Ultrasensitive Mouse Insulin kit; Mercodia, Uppsala, Sweden).
Histology and villus length along the small intestinal axis. Morphology of proximal, mid and distal small intestine was assessed by hematoxylin and eosin staining of formalin-fixated material. Proliferating cells were detected by staining of nuclear Ki-67 antigen. Morphometrical analysis of small intestinal samples was performed as described by evaluation of 4, 6 and 7 villi per proximal, mid and distal intestinal segment, respectively, of 4 to 6 animals per group. The digitized images were evaluated at 10 x magnification using the calibrated image analysis system (Leica Quantimet 570 C; Leica Qwin Pro V 2.8). The epithelial surface lining was demarcated and measured as a parametrical length, whereby 1 pixel was equal to 0.544 µm.
Glucose/lactose absorption. The analysis of $^{13}C_6$-glucose and ^{13}C-glucose concentrations from blood spots was performed according to Van Dijk *et al.* by gas chromatography-mass spectrometry (SSQ700, ThermoFischer B.V., Breda, The Netherlands) [25].
Disaccharidase activity assay in mucosal homogenates. A portion of small intestinal mucosa (from the proximal, mid and distal part) was homogenized with PBS buffer containing protease inhibitors (Roche, Indianapolis, USA) in order to make 4% homogenates for use in enzyme activity assay. Enzyme activity level of lactase was measured in freshly scraped intestinal mucosa as described previously by Dahlqvist [28]. Activity levels were normalized to protein levels, measured by the BCA method as described by the manufacturer (Pierce, Rockford, IL).
Measurement of mRNA expression by real-time PCR (Taqman). mRNA expression of the differentiation marker and lactose digesting enzyme (lactase) was measured in proximal, mid and distal small intestine by real-time PCR as described previously [29]. In addition, mRNA expression levels of intestine-specific transcription factors (Cdx-2, Gata-4 and Hnf-1α) were measured by real-time PCR in the mid part of the small intestine. PCR results were normalized to β-actin mRNA levels. The sequences of the primers and probes are listed in Table 1.

Table 1. Primer and probe sequences

Gene	GenBank	Forward Primer	Reversed Primer	TaqMan® probe
β-actin	NM_007393	AGC CAT GTA CGT AGC CAT CCA	TCT CCG GAG TCC ATC ACA ATG	TGT CCC TGT ATG CCT CTG GTC GTA CCA C
Lactase	XM_129479	CGT CTG CTT CCT ATC AGG TTG AA	GTG GGA AAA TGT GTC CCA GAT ACT	TCC TTT GCC ATC TGC TCT CCA CGC
Cdx-2	NM_007673	GTC CCT AGG AAG CCA AGT GAA A	CTC CTG ATG GTG ATG TAT CGA CTA	CCT TCT CCA GCT CCA GCC GCT G
Gata-4	NM_008092.2	GAG ATG CGC CCC ATC AAG	GAC ACA GTA CTG AAT GTC TGG GAC AT	CTG TCA TCT CAC TAT GGG CAC AGC AGC TC
Hnf-1α	NM_009327	CTC CAG CAG CCT GGT GTT GT	GAG GCC ATC TGG GTG GAG AT	CAC AGC CAC CTG CTG CCA TCC AAC

LA determination in phospholipids of intestinal mucosa. Thirty mg of intestinal mucosa was homogenized in 200 µl of 0.9% NaCl and lipids were extracted according to Bligh and Dyer after the addition of the fatty acid internal standard (C17:0) and anti-oxidant (BHT) [30]. Lipid extracts were fractionated into phospholipids and other lipids using TLC (20 x 20 cm, Silica gel 60 F254; Merck) with hexane/diethyl ether/acetic acid (80:20:1, v:v:v) as running solvent. Phospholipid spots were scraped and phospholipids were extracted by methanol/chloroform. Phospholipid LA ratio was determined according to Muskiet *et al.* as described previously [24,31].

Statistical analysis

Values represent means ± SD for the indicated number of mice per group. Using SPSS version 12.0.2 statistical software (Chicago, IL, USA), we calculated significance of differences with the Mann-Whitney *U*-test. P-values below 0.05 were considered statistically significant.

RESULTS

Body weight and food ingestion were assessed every two weeks and there were no significant differences in basal or final body weight, nor in food intake (data not shown) between EFA-deficient and control mice.

Pronounced EFA deficiency of EFA-deficient mice

After eight weeks of treatment, in mice fed the EFA-deficient diet, the triene/tetraene ratio in red blood cell membranes was strongly increased compared with the control group (0.23 ± 0.06 vs. 0.01 ± 0.00; respectively, $p<0.01$). Conform previous observations, the mice fed the EFA-deficient diet also showed other characteristics of EFA deficiency including increased bile flow (+78%, $p<0.05$), biliary secretion rates of bile salts (+212%, $p<0.01$) and phospholipids (+82%, $p<0.05$), and higher levels of triene/tetraene ratio in plasma (0.55 ± 0.20 vs. 0.01 ± 0.00, $p<0.01$), compared with control mice (data not shown) [4]. Fat balance during 72 hours revealed a decreased total fat absorption in EFA-deficient compared to control mice (81% vs. 99%, resp., $p<0.01$; fig 1). The absorption of saturated fatty acids, palmitic (C16:0) and stearic (C18:0) acids, was affected to a greater extent than that of the unsaturated fatty acids oleic (C18:1n-9) and linoleic acid (C18:2n-6). Together, these observations indicated that the mice fed the EFA-deficient diet had profound EFA deficiency after 8 weeks on the experimental diet [4].

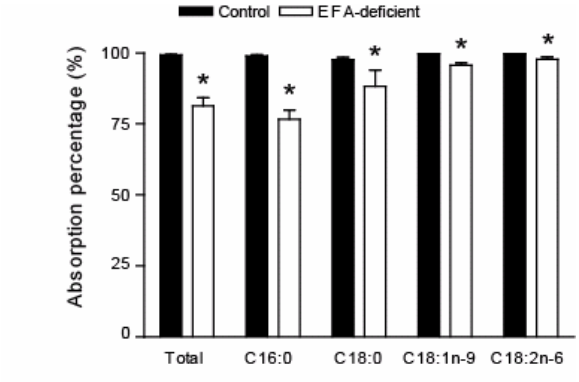

Figure 1. Fat absorption of total dietary fat, and of major dietary fatty acids (16:0, 18:0, 18:1n-9 and 18:2n-6) in EFA-deficient and control mice. Feces were collected after a 72h period in which the food intake was monitored by weighing food containers. Absorption was calculated by subtracting fecal excretion of these fatty acids after 72h from their dietary intake in 72h and then multiplying the result by 100. Data represent means ± SD of 7 mice per group. **P*< 0.05 and ***P*< 0.01 for EFA-deficient vs. control mice.

EFA deficiency in mice not associated with alterations in intestinal morphology

Hematoxylin/eosin (data not shown) and Ki67 staining of the three segments of the small intestine revealed no clear differences in morphology or proliferative capacity between EFA-deficient and control mice (fig 2). The villus lengths were similar in EFA-deficient and control mice, as determined by morphometrical measurements in the three segments of the small intestine (fig 2).

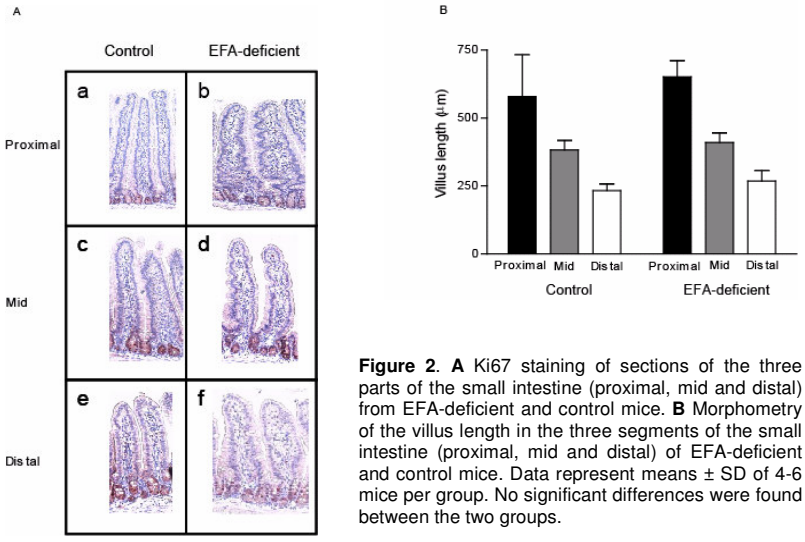

Figure 2. **A** Ki67 staining of sections of the three parts of the small intestine (proximal, mid and distal) from EFA-deficient and control mice. **B** Morphometry of the villus length in the three segments of the small intestine (proximal, mid and distal) of EFA-deficient and control mice. Data represent means ± SD of 4-6 mice per group. No significant differences were found between the two groups.

EFA deficiency is associated with delayed glucose clearance

Basal blood glucose concentrations were similar in EFA-deficient and control mice. After intragastric administration of the glucose/lactose bolus, glucose concentrations rapidly increased in control mice, with a maximum concentration at 30 min. after administration (fig 3). In EFA-deficient mice, the increase in blood glucose levels was similar to that in control mice up to 30 min., but then continued to increase, reaching a maximum concentration at 60 min. The glucose concentrations between 60 and 180 min. were slightly, but significantly higher in EFA-deficient mice compared to controls (+10-15%, $p<0.05$). Accordingly, the area under the curve was higher for the EFA-deficient mice compared with controls (+15%,

p<0.05, data not shown). Based on the apparently delayed glucose clearance, we determined insulin concentrations at the end of the experiment (at ~180 min.). In EFA-deficient mice insulin concentrations were significantly higher than in control mice (0.55 µg/ml vs. 0.35 µg/ml, respectively, p<0.01).

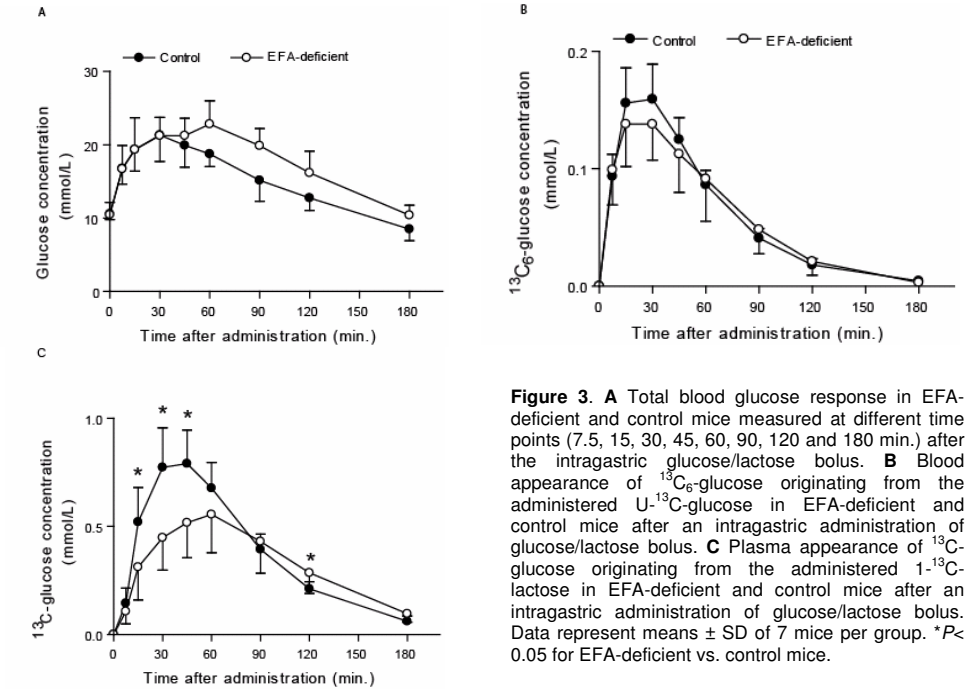

Figure 3. **A** Total blood glucose response in EFA-deficient and control mice measured at different time points (7.5, 15, 30, 45, 60, 90, 120 and 180 min.) after the intragastric glucose/lactose bolus. **B** Blood appearance of $^{13}C_6$-glucose originating from the administered U-^{13}C-glucose in EFA-deficient and control mice after an intragastric administration of glucose/lactose bolus. **C** Plasma appearance of ^{13}C-glucose originating from the administered 1-^{13}C-lactose in EFA-deficient and control mice after an intragastric administration of glucose/lactose bolus. Data represent means ± SD of 7 mice per group. **P*< 0.05 for EFA-deficient vs. control mice.

Similar glucose absorption but delayed lactose digestion in EFA-deficient mice

To assess the competence of monosaccharide absorption in EFA deficiency, we determined blood appearance of $^{13}C_6$-glucose (fig 3). After the administration of the bolus, blood $^{13}C_6$-glucose concentration rapidly increased with a maximum at 45 min. for both groups. After 45 min., $^{13}C_6$-glucose rapidly disappeared until 120 min., after which the rate of disappearance decreased in both EFA-deficient and control mice. Thus, the blood $^{13}C_6$-glucose appearance and disappearance was similar in EFA-deficient and control mice, supporting unaffected monosaccharide absorption in the former.

In order to measure the competence of disaccharide digestion and absorption, we determined blood appearance of ^{13}C-glucose, originating from the administered 1-^{13}C-lactose (fig 3). ^{13}C-glucose reached a maximum concentration in control mice at 45 min. after bolus administration. The ^{13}C-glucose disappeared from the blood within the next 2 hours, with the slowest disappearance during the last hour after the bolus administration. Blood appearance of ^{13}C-glucose in EFA-deficient mice, however, increased to a slower extent and reached its maximal concentration at approximately 60 min. after the bolus administration. Thus, the ^{13}C-lactose uptake was delayed in EFA-deficient compared to control mice.

Decreased mRNA expression and lactase activity in mid small intestine of EFA-deficient mice

Lactase is a critical disaccharidase during early postnatal life and a sensitive intestinal marker for functional changes occurring in the small intestine of the adult animal. Its activity relatively decreases during weaning to low adult levels, thus the total lactase activity remains the same during the adutlhood [32]. Measurement of the enzyme activity of lactase along the proximal-to-distal axis of the small intestine revealed a lower activity in the mucosa of the mid part of the small intestine of EFA-deficient compared to control mice (fig 4). The decreased lactase activity corresponded with lower mRNA levels of lactase, as shown by quantitative PCR (fig 4). We determined if reduced lactase mRNA expression levels were regulated at the transcriptional level. However, the mRNA expression of transcription factors involved in regulation of the lactase mRNA expression, namely Cdx-2, Gata-4 and Hnf-1α (fig 4), was not different between EFA-deficient and control animals.

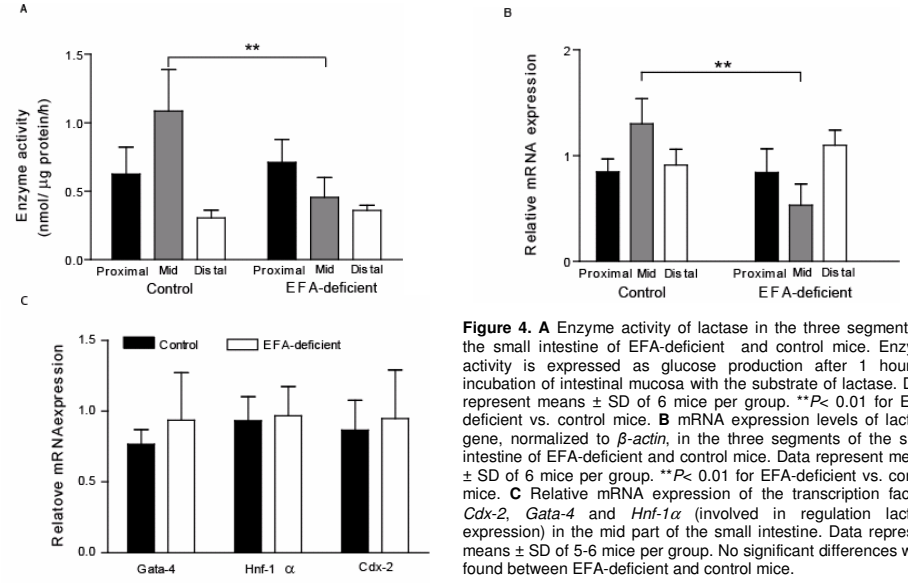

Figure 4. A Enzyme activity of lactase in the three segments of the small intestine of EFA-deficient and control mice. Enzyme activity is expressed as glucose production after 1 hour of incubation of intestinal mucosa with the substrate of lactase. Data represent means ± SD of 6 mice per group. $^{**}P< 0.01$ for EFA-deficient vs. control mice. **B** mRNA expression levels of lactase gene, normalized to *β-actin*, in the three segments of the small intestine of EFA-deficient and control mice. Data represent means ± SD of 6 mice per group. $^{**}P< 0.01$ for EFA-deficient vs. control mice. **C** Relative mRNA expression of the transcription factors *Cdx-2*, *Gata-4* and *Hnf-1α* (involved in regulation lactase expression) in the mid part of the small intestine. Data represent means ± SD of 5-6 mice per group. No significant differences were found between EFA-deficient and control mice.

Decreased lactase activity and mRNA expressions are associated with low LA concentrations in the mid small intestine

EFAs are involved in regulation of membrane fluidity and alterations in membrane lipid matrix. Therefore, it has been proposed that EFAs indirectly influence normal conformation and functioning of the proteins embedded in the inner and/or outer leaflet of the membrane [14]. For this reason we tested if lactase activity in the mid segment of the small intestine correlated with LA levels. Phospholipid LA concentration was determined the mucosa of the three segments of the small intestine (fig 5). LA concentrations were highest in the mid part of the small intestine in control mice. Interestingly, LA concentration was significantly lower in the mid part of the small intestinal mucosa of EFA-deficient compared to control mice (26 mol% vs. 16 mol%, respectively, $p<0.01$). LA concentrations in proximal and distal part were similar in both groups. In the mid small intestine LA concentrations positively correlated with lactase activity ($r=0.88$, $p<0.001$) and mRNA expression of lactase ($r=0.79$, $p<0.01$) (fig 5). Decreased mRNA levels in the mid intestine indicate that the intestinal impairment can not exclusively be the result of alterations in membrane composition and fluidity.

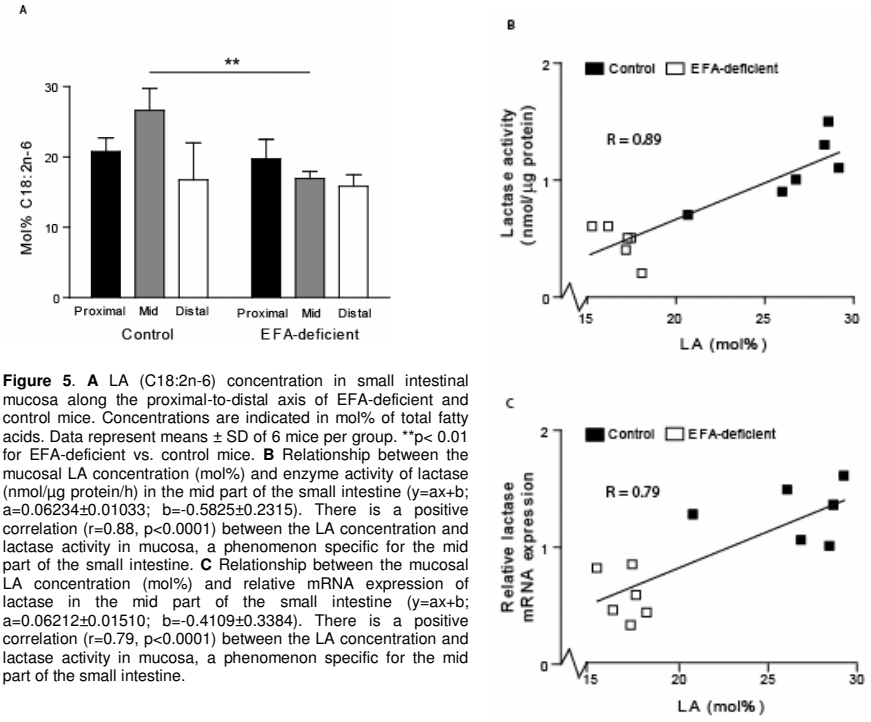

Figure 5. **A** LA (C18:2n-6) concentration in small intestinal mucosa along the proximal-to-distal axis of EFA-deficient and control mice. Concentrations are indicated in mol% of total fatty acids. Data represent means ± SD of 6 mice per group. **p< 0.01 for EFA-deficient vs. control mice. **B** Relationship between the mucosal LA concentration (mol%) and enzyme activity of lactase (nmol/µg protein/h) in the mid part of the small intestine (y=ax+b; a=0.06234±0.01033; b=-0.5825±0.2315). There is a positive correlation (r=0.88, p<0.0001) between the LA concentration and lactase activity in mucosa, a phenomenon specific for the mid part of the small intestine. **C** Relationship between the mucosal LA concentration (mol%) and relative mRNA expression of lactase in the mid part of the small intestine (y=ax+b; a=0.06212±0.01510; b=-0.4109±0.3384). There is a positive correlation (r=0.79, p<0.0001) between the LA concentration and lactase activity in mucosa, a phenomenon specific for the mid part of the small intestine.

DISCUSSION

Our previous studies suggested that EFA deficiency in mice affects fat absorption at the level of the small intestinal mucosa [4]. We now explored the effects of EFA deficiency in mice on mucosal histology and on a physiological function of the small intestine, carbohydrate digestion and absorption. Our data demonstrate that EFA deficiency is not only associated with fat malabsorption, but also with impaired lactose digestion in the murine model of EFA deficiency. The impaired lactose digestion coincided with an ~50% reduced lactase activity and mRNA expression in mid small intestine of EFA-deficient mice. Intestinal lactase activity and mRNA expression strongly correlated with mucosal linoleic acid concentrations, which were depressed in EFA deficiency, particularly in mid-intestine.

As expected from previous studies, our murine model of EFA deficiency was clearly deficient, as indicated by elevated triene/tetraene ratios in erythrocytes and plasma, fat malabsorption, and other biochemical signs of EFA deficiency, like increased bile flow and biliary output [4]. EFA deficiency in mice did not affect morphology or proliferative capacity of the small intestine. As far as we know, our study is the first to describe the effects of EFA deficiency on the intestinal morphology in mice. Christon *et al.* have shown that low dietary linoleic acid levels were associated with alterations in villi and crypt sizes in rats [33]. We did not observe differences in villus length between EFA-deficient and control mice using morphometrical evaluation of the villus length in the proximal-to-distal axis of the small intestine. These results indicate that EFA deficiency associated malabsorption of fats and disaccharides is not associated with morphological alterations in small intestine of mice.

To assess small intestinal function in EFA-deficient mice, we studied carbohydrate absorption, using stable isotope methodology [25]. The advantage of stable isotope methodology is that it can easily be extrapolated to patient studies [21,22]. EFA-deficient mice

had higher total blood glucose levels from 60 min. after the administration of the glucose/lactose bolus. High total glucose levels in blood could theoretically be explained by lower blood glucose clearance (slower postprandial uptake of glucose by the peripheral tissues), rather than by disturbed intestinal absorption. This hypothesis is supported by higher insulin concentrations at the end of the experiment in EFA-deficient compared with control mice. This observation is in accordance with previous studies suggesting a relationship between EFA deficiency and insulin resistance [34]. However, we cannot exclude that the increased content of saturated fats in the EFA deficient diet contributes to this phenomenon, independently from EFA deficiency [35].

Measurement of the absorption of $^{13}C_6$-glucose, originating from the administered U-^{13}C-glucose, revealed similar appearance and disappearance of the labeled glucose in both groups. This observation indicates that EFA deficiency does not affect the absorption of the monosaccharide glucose in mice. The blood appearance of ^{13}C-glucose originating from lactose, however, was significantly delayed in EFA-deficient mice. The discrepancy in the effect of EFA deficiency on glucose and lactose absorption could be explained by the diverse intestinal fates of these carbohydrates. Unlike glucose, which is directly transported by the glucose transporters across the brush border membrane of the enterocyte, lactose first needs to be hydrolyzed by the enzyme lactase [7,8]. In order to investigate whether our functional results corresponded with altered lactase activity or expression, we measured these parameters in EFA-deficient and control mice. Lactase is the critical enzyme for hydrolysis of lactose and a good marker of functional changes in the small intestine [10]. The delayed lactose digestion corresponded with an approximate 50% reduction in both lactase activity and mRNA expression compared to control mice. The mRNA levels of relevant transcription factors for lactase mRNA expression were unaffected in EFA-deficient mice, suggesting that the expression of lactase is regulated in a post-transcriptional manner during EFA deficiency.

Under physiological conditions phospholipids of the small intestinal mucosa contain considerable amounts of LA (C18:2n-6) and of its long-chain polyunsaturated fatty acid metabolite arachidonic acid (AA, C20:4n-6) [36]. During EFA deficiency the levels of this major dietary EFA are decreased in intestinal mucosa [12,37]. We observed LA deficiency in mucosal phospholipids, particularly the mid part of the small intestinal mucosa, which strongly correlated with reduced lactase activity and mRNA expression. We speculate that the decreased levels of LA in this intestinal segment correspond to the predominant location of nutrient absorption [38]. It is tempting to speculate that low levels of LA in phospholipids of cellular membranes lead to structural and physiological changes in the lipid membrane, eventually causing functional changes in membrane anchoring lactase enzyme. Since not only lactase activity but also its mRNA expression was decreased in EFA deficiency, it is likely that altered membrane fluidity is not the single factor involved.

Our present results indicate that EFA deficiency has functional consequences for small intestinal function in mice, and it provides indirect support for the hypothesis that reduced mucosal function is involved in fat malabsorption in EFA deficiency. EFA deficiency in (pediatric) cholestatic patients seems primarily caused by fat malabsorption due to bile deficiency. Recently, we reported that cholestasis *per se* does not affect carbohydrate digestion or absorption, using a rat model of short-term cholestasis [39]. Our present study indicates, however, that EFA deficiency aggravates the malabsorption of fat, and decreases the small intestinal capacity to digest carbohydrates. Decreased levels of LA in the mid part of the small intestine seem to play a pathophysiological role in the diminished mucosal

function in EFA deficiency. Our findings imply that the nutrition of cholestatic patients encountering EFA deficiency should accommodate the decreased capacity to absorb fat, the EFA deficiency (possibly by using LA-rich phospholipids) [40], and the reduced capacity to digest disaccharides.

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CHAPTER 6

Essential fatty acid deficiency in mice: milder fat malabsorption and a more hydrophobic bile salt composition

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In preparation

ABSTRACT

Cholestatic liver disease is frequently accompanied by fat malabsorption and essential fatty acid (EFA) deficiency. EFA deficiency in mice disturbs fat malabsorption via unidentified mechanisms, presumably at the level of the intestinal mucosa. The farnesoid X receptor (FXR) is involved in the regulation of bile salt homeostasis in the enterohepatic circulation. We addressed the role of FXR in fat absorption and bile salt homeostasis in EFA deficient mice.

Fxr$^{-/-}$ and *Fxr*$^{+/+}$ (control) mice were fed an EFA deficient diet for 8 weeks, after which fat absorption was determined by 72 h fat balance. Bile production and intestinal mRNA expression of proteins relevant for bile salt homeostasis and lipid homeostasis were analyzed.

EFA deficient diet induced a similar degree of EFA deficiency in *Fxr*$^{-/-}$ and *Fxr*$^{+/+}$ mice (triene/tetraene ratio: 0.14 (median, range 0.09-0.32) vs. 0.13 (0.07-0.24), resp. Fat absorption, however, was significantly better preserved in *Fxr*$^{-/-}$ mice (78±4% of amount ingested) compared with controls (70±4%, $P<0.05$). Correspondingly, *Fxr*$^{-/-}$ mice gained more body weight during the experimental period compared with controls (+14±7 vs. +7±5% of initial body weight, respectively; $P<0.05$). Bile flow and biliary secretion rates of bile salts, cholesterol and phospholipids were similar in *Fxr*$^{-/-}$ and control mice. The composition of the biliary bile salt pool was altered in *Fxr*$^{-/-}$ mice, however, characterized by increased hydrophobicity (cholic acid-muricholic acid ratio: 1.5 (0.93-4.21) *vs.* 1.0 (0.34-1.12), $P<0.05$). *Fxr*$^{-/-}$ mice had a higher fecal bile salt loss (+60%, $P<0.01$), coinciding with a lower intestinal mRNA expression of bile salt transporter Asbt in the terminal ileum.

We conclude that inactivation of FXR ameliorates the fat malabsorption and improves growth of EFA deficient mice, probably by increasing the hydrophobicity of the bile salt pool.

INTRODUCTION

Cholestasis is defined as a decreased flow of bile and its consituents into the small intestine. Biliary bile salts aid in the absorption of lipids and lipid-soluble vitamins from the intestine. Consequently, cholestatic liver disease is frequently accompanied by fat malabsorption and essential fatty acid (EFA) deficiency, eventually leading to malnutrition. Malnutrition seriously worsens the prognosis and treatment outcome in cholestatic children [1-3].

The fatty acids linoleic (LA; C18:2n-6) and linolenic (ALA; C18:3n-3) acid cannot be synthesized *de novo*. These so-called essential fatty acids therefore need to be acquired from external sources, usually the diet. After absorption, linoleic LA and ALA can be converted into long chain polyunsaturated fatty acids (LCPUFAs) such as arachidonic (AA; C20:4n-6), eicopentaenoic (EPA; C20:5n-3) and docosahexaenoic (DHA; C22:6n-3) acid. Deficiency of EFAs and LCPUFAs has been associated with obesity, hypertension, diabetes mellitus, schizophrenia, Alzheimer's disease and cancer [4,5].

Not only cholestasis can induce fat malabsorption, EFA deficiency in itself also causes fat malabsorption in rats and mice [5]. The mechanism by which EFA deficiency, in the absence of cholestasis, decreases fat absorption is incompletely understood. EFA deficiency-induced fat malabsorption in rats has been ascribed to decreased bile formation, impaired triglyceride re-esterification and impaired chylomicron formation [6,7]. EFA deficiency in mice, however, is accompanied by increased bile formation [8].

The farnesoid X receptor (FXR) has been implicated in the regulation of bile salt and lipid metabolism [9]. Fxr-deficient mice display increased bile flow and bile salt pool size, and a more hydrophobic bile salt composition due to an increased contribution of cholic acid (CA) [8,10]. Interestingly, we previously demonstrated that EFA-deficiency in (wild-type) mice has similar phenotypic characteristics [8]. Based on this similarity, we addressed whether FXR inactivation would affect the phenotype of EFA deficiency in mice. We hypothesized that EFA deficiency in Fxr-null mice would ameliorate fat malabsorption, compared with control mice, by (further) increasing bile flow, bile salt pool size and relative CA contribution.

MATERIAL AND METHODS

Animals and housing

We used Fxr knockout mice that were originally described by Kok *et al.* [10]. Male homozygous (*Fxr*$^{-/-}$) and wild-type (*Fxr*$^{+/+}$) mice (C57BL/6J-129/OlaHsd; 25-35 g) were bred at the animal facility of the University of Groningen. Mice were housed in a light- and temperature-controlled facility. Food and water were available *ad libitum*. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, Netherlands.

Experimental diet

We used high-fat EFA-deficient (16 wt% and 34 energy% fat) to induce EFA deficiency in mice. The diet was custom synthesized by Arie Bloks BV (Woerden, the Netherlands, diet code: #4141.08). The EFA-deficient diet contained 70 mol% palmitic acid (C16:0), 19 mol% stearic acid (C18:0), 9 mol% oleic acid (C18:1n-9) and 2 mol% linoleic acid (C18:2n-6). Fatty acid contents of the diets were analyzed by extracting, hydrolyzing and methylating total dietary fatty acids as described by Muskiet *et al.* and subsequent separation and

quantification of fatty acid methyl esters was performed by gas chromatograhy as described previously [8,11].

Experimental procedures

Mice were fed standard laboratory chow containing 6 weight% fat from weaning, and switched to an EFA deficient high-fat (16 weight%) diet at 8 wk of age. At the end of an 8 wk-period on EFA deficient diet, feces were collected over 72 hours to measure fat balance. Bile production was determined by bile collection for 30 min via cannulation of the gallbladder. After bile collection, mice were sacrificed by obtaining a large blood sample via cardiac puncture. Erythrocyte EFA status was assessed by the triene/tetraene ratio, obtained from gaschromatography of fatty acid methyl esters [12]. The small intestine was excised, flushed with ice-cold PBS and was divided into 3 pieces of equal length. Material was harvested for gene expression from the middle of each piece and the distal end of the third piece, representing the proximal, medial, distal and terminal ileal segment of in the intestine.

Analytical methods

The erythrocyte triene/tetraene ratio was determined as described by Werner *et al.* [12]. Fat absorption was determined by quantification of fatty acid ingestion and fecal excretion over a 72 h period, using gas chromatography [13]. Bile salt composition of bile and fecal excretion of bile salts were determined [14]. Plasma and biliary cholesterol and phospholipids, and plasma triglyceride concentrations were determined by routine laboratory techniques.

RNA isolation and measurement of mRNA levels by real-time PCR (Taqman)

mRNA expression levels in proximal, medial, distal and terminal ileal part of the small intestine were measured by real-time PCR, as described previously [15]. PCR results were normalized to β-actin mRNA levels. The sequences of the primers and probes are listed in Table 1.

Table 1. Primer and probe sequences

Gene	GenBank	Forward Primer	Reversed Primer	TaqMan® probe
β-actin	NM_007393	AGC CAT GTA CGT AGC CAT CCA	TCT CCG GAG TCC ATC ACA ATG	TGT CCC TGT ATG CCT CTG GTC GTA CCA C
Fat	BC010262	GAT CGG AAC TGT GGG CTC AT	GGT TCC TTC TTC AAG GAC AAC TTC	AGA ATG CCT CCA AAC ACA GCC AGG AC
Fatp-4	NM_011989	CCA GAC AAG GGT TTT ACA GAT AAG CT	ACC TGC TGT GCA CCA CAA TG	CGG GCA CCA CGG GGC TAC CC
Ifabp	NM_007980	GAG TTG AGG CCA AGC GAT TCT	GAG CCT GGC ATT AGC ATG ATG	CTC TTC AGC GTT GCT CCA GGC TCT GAG
Dgat-1	NM_010046.2	GGT GCC CTG ACA GAG CAG AT	CAG TAA GGC CAC AGC TGC TG	CTG CTG CTA CAT GTG GTT AAC CTG GCC A
Dgat-2	NM_026384.2	GGG TCC AGA AGA AGT TCC AGA AG	CCC AGG TGT CAG AGG AGA AGA G	CCC CTG CAT CTT CCA TGG CCG
Mttp	NM_008642	CAA GCT CAC GTA CTC CAC TGA AG	TCA TCA TCA CCA TCA GGA TTC CT	ACC GCA AGA CAG CGT GGG CTA CA
Fxr-α	U09417	CTT TCT GAA AGC TTA TTT GGT ATG CTA A	AGT ACG ATT CCA AAT CCA GAT TCT G	AAC ACG CGG CAG GCC CTC TG
Fxr-β	AK002513	GTG AAG CCA GCT AAA GGT ATG CTA A	AGT ACG ATT CCA AAT CCA GAT TCT G	AAC ACG CGG CAG GCC CTC TG
Fgf-15	NM_008003	GCC ATC AAG GAC GTC AGC A	CTT CCT CCG AGT AGC GAA TCA G	CGC TCA TGC AGA GGT ACC GCA CG
Asbt	NM_011388	ACC ACT TGC TCC ACA CTG CTT	CCC GAG TCA ACC CAC ATC TT	CCC TTG GAA TGA TGC CTC TTT GCC TC
Ibabp	NM_008375	CCC CAA CTA TCA CCA GAC TTC G	ACA TCC CCG ATG GTG GAG AT	TCC ACC AAC TTG TCA CCC ACG ACC T

Statistical analysis

Results are provided in means ± SD or median and range for the indicated number of mice per group. Using SPSS version 12.0.2 statistical software (Chicago, IL, USA), we analyzed the results with the two-tailed Student's t-test for normally distributed data or with the Mann-Whitney *U*-test for data that were not normally distributed. Variance among data was determined using Levene's test for equality of variances. $P<0.05$ was considered significant.

RESULTS

EFA deficiency in *Fxr*$^{-/-}$ mice: higher weight gain and fat absorption

EFA deficiency was induced in *Fxr*$^{-/-}$ and *Fxr*$^{+/+}$ mice to a similar extent as indicated by the triene/tetraene ratio measured in RBC (median (range): 0.14 (0.09-0.32) vs. 0.13 (0.07-0.24), resp., NS). After 8 weeks on an EFA deficient diet, *Fxr*$^{-/-}$ mice had gained more weight compared to *Fxr*$^{+/+}$ mice (+14±7 vs. +7±5% of initial body weight, $P<0.05$). Fat ingestion was similar in *Fxr*$^{-/-}$ and *Fxr*$^{+/+}$ mice, while fecal fat excretion was lower in *Fxr*$^{-/-}$ mice (197±56 vs. 268±53 µmol/day, $P<0.05$; Fig 1). Absorption coefficients of total fatty acids, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9) and linoleic acid (C18:2n-6) were higher in *Fxr*$^{-/-}$ mice compared to *Fxr*$^{+/+}$ mice (all $P<0.05$; Fig 1).We quantified the mRNA expression of proteins that have been implicated in fatty acid transport, including fatty acid translocase (Fat), fatty acid transport protein 4 (Fatp-4), and cytosolic intestinal fatty acid binding protein 1 (Ifabp-1). mRNA expression of *Fat*, *Ifabp-1* and *Fatp-4* was not changed in *Fxr*$^{-/-}$ mice. *Fat* and *Fatp-4* expression was significantly higher in proximal and medial part compared to the distal part of the small intestine in both groups ($P<0.01$), similar to previous reports [16-18]. *Ifabp-1* expression was higher in the medial part compared to the proximal and distal part of the small intestine in the *Fxr*$^{-/-}$ mice ($P<0.01$; Fig 1).

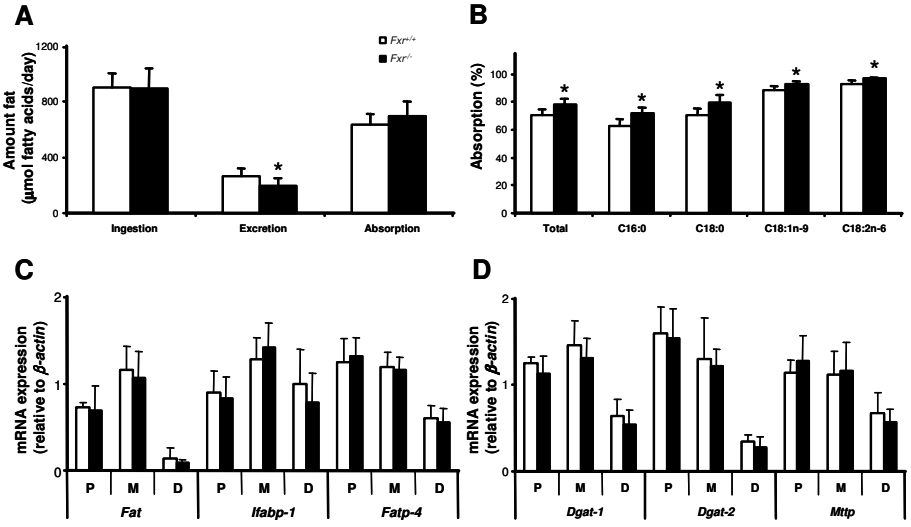

Figure 1. (A) Fat ingestion, fecal excretion and net absorption measured over a 72h period in EFA deficient Fxr$^{+/+}$ (white bars) and Fxr$^{-/-}$ mice (black bars). (B) Absorption percentages of total fatty acids and of the major fatty acids: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9) and linoleic acid (C18:2n-6). (C) mRNA expression of *Fat*, *Fatp-4* and *Ifabp*, normalized to *β-actin* levels, in the proximal (P), medial (M) and distal (D) part of the small intestine of EFA deficient *Fxr*$^{+/+}$ (white bars) and *Fxr*$^{-/-}$ mice (black bars), measured by quantitative PCR. (D) mRNA expression of *Dgat-1*, *Dgat-2* and *Mttp*, normalized to *β-actin* levels, in the proximal (P), medial (M) and distal (D) part of the small intestine of EFA deficient *Fxr*$^{+/+}$ (white bars) and *Fxr*$^{-/-}$ mice (black bars), measured by quantitative PCR. *Fxr*$^{-/-}$ mice; n=7 and *Fxr*$^{+/+}$ mice; n=5. Results are expressed as means ± SD. **P*<0.05.

The enzymes involved in re-esterification of triglycerides in the enterocyte include acyl-CoA:diacylglycerol acyltransferase (DGAT) 1 and 2, catalyzing the esterification of diacylglycerol to triglycerides. Microsomal triglyceride transfer protein (Mttp) is essential for the proper assembly of cholesterol, triglycerides, phospholipids and apolipoprotein B into chylomicrons [19,20]. Expression of *Dgat-1*, *Dgat-2* and *Mttp* showed higher expression in the proximal and medial part compared to the distal part (P<0.01; Fig 1), similar to previous reports [21,22]. Inactivation of FXR did not alter this expression pattern.

EFA deficiency in *Fxr*$^{-/-}$ mice increases plasma cholesterol and phospholipids concentration

Plasma triglycerides were slightly, but not significantly higher in EFA deficient *Fxr*$^{-/-}$ mice, compared with controls (0.6±0.2 vs. 0.4±0.0 mmol/L, NS). Plasma concentrations of cholesterol (+31%, P<0.01) and phospholipids (+38%, P<0.05) were increased in EFA deficient in *Fxr*$^{-/-}$ compared to EFA deficient control mice (Fig 2).

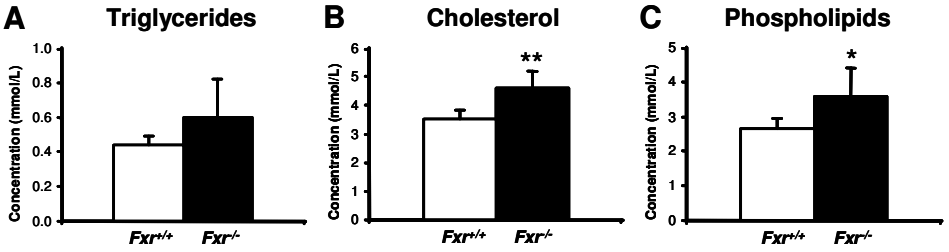

Figure 2. Plasma concentrations of (A) triglycerides, (B) cholesterol, and (C) phospholipids in EFA deficient *Fxr*$^{+/+}$ (white bars, n=5) and *Fxr*$^{-/-}$ mice (black bars, n=7). Results are expressed as means ± SD. **P*<0.05 and ***P*<0.01.

Bile salt homeostasis and enterohepatic circulation

Figure 3 shows that bile production parameters were similar in EFA deficient *Fxr*$^{-/-}$ and control mice. Bile flow was also similar in *Fxr*$^{-/-}$ and control mice (5.5±1.1 vs. 6.5±1.5 µL/min/100 g body weight, respectively, as was the biliary bile salt output (258±104 vs. 239±117 nmol/min/100 g body weight, respectively). *Fxr*$^{-/-}$ mice fed a regular (not EFA-deficient) diet and wild type EFA deficient mice both have an increased contribution of CA to total bile salt composition [8,10]. Figure 4 shows the result of the combination, EFA deficient *Fxr*$^{-/-}$ mice; the contribution of CA to total bile salts was higher in EFA deficient *Fxr*$^{-/-}$ mice, at the expense of β-muricholic acid (β-MA) and α-muricholic acid (α-MA), resulting in a increased cholic acid-muricholic acids ratio, compared with controls [median 1.5, (range 0.93-4.21) vs. 1.0 (0.34-1.12), P<0.05].

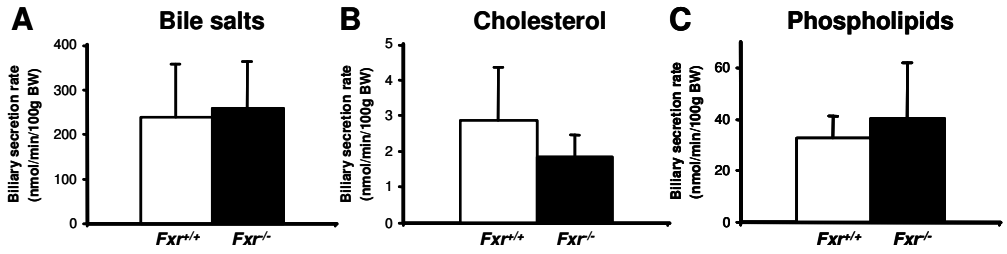

Figure 3. Biliary secretion rates of (A) bile salts, (B) cholesterol, and (C) phospholipids in EFA deficient *Fxr*$^{+/+}$ (white bars, n=5) and *Fxr*$^{-/-}$ mice (black bars, n=7). Results are expressed as means ± SD.

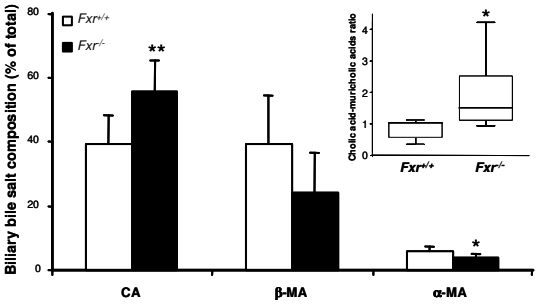

Figure 4. Biliary bile salt composition in EFA deficient *Fxr*$^{+/+}$ (white bars, n=5) and *Fxr*$^{-/-}$ mice (black bars, n=7). Expressed are cholic acid (CA), β-muricholic acid (β-MA) and α-muricholic acid (α-MA) as a percentage of the total bile salt concentration. Inset: cholic acid-muricholic acids ratio. Results are expressed as means ± SD. **P*<0.05 and ***P*<0.01.

Fecal bile salt excretion is increased in EFA deficient *Fxr*$^{-/-}$ mice

EFA-deficiency increased fecal bile salt excretion in *Fxr*$^{-/-}$ mice (3.6±0.6 vs. 2.3±0.4 μmol/day, *P*<0.01), coinciding with decreased Asbt mRNA expression (-30%; P<0.05). Fxr deficiency decreased the expression of the FXR target genes *Fgf-15* and *Ibabp* in EFA deficient mice, but this reached statistical significance only in the latter (Fig 5).

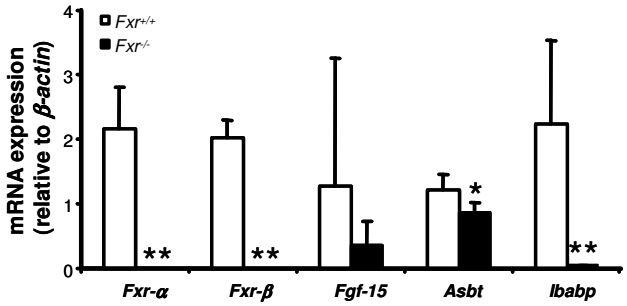

Figure 5. mRNA expression of Fxr and relevant Fxr target genes in the terminal ileum in EFA deficient *Fxr*$^{+/+}$ (white bars, n=5) and *Fxr*$^{-/-}$ mice (black bars, n=7). (A) mRNA levels were determined of *Fxr-α*, *Fxr-β*, *Fgf-15*, *Asbt* and *Ibabp*, all normalized to *β-actin*, using quantitative PCR. Results are expressed as means ± SD. **P*<0.05 and ***P*<0.01.

DISCUSSION

We addressed to what extent the gastrointestinal phenotype, i.e. fat malabsorption and bile salt homeostasis, of EFA deficiency was influenced by FXR inactivation in mice. Our data show that inactivation of FXR renders mice more resistant to EFA deficiency induced fat malabsorption and increases their weight gain, possibly by the production of bile with a more hydrophobic bile salt composition.

For our study, we used the *Fxr*$^{-/-}$ mice originally described by Kok *et al.* [10]. The complete abolishment of *Fxr-α* and *Fxr-β* expression (Fig 5) in the *Fxr*$^{-/-}$ mice confirmed that the mice did not express the Fxr gene. Kok *et al.* demonstrated that chow-fed *Fxr*$^{-/-}$ mice had an increased bile salt pool size and an increased fecal bile salt loss [10]. This observation was explained by the abolishment of the negative feedback regulation that FXR exerts on hepatic bile salt synthesis under physiological conditions. Sinal *et al.,* however, reported a decreased bile salt pool and fecal bile salt loss in another *Fxr*$^{-/-}$ mouse model [23]. It is tempting to speculate that the difference between the two mouse models is due to the fact that the *Fxr*$^{-/-}$ mice used in Sinal's study still have a DNA-binding domain which could affect the expression FXR target genes.

Similar to FXR inactivation, EFA deficiency in mice increases bile salt pool size, bile flow and biliary output rate of bile salts [8,10]. Based on this information, we expected that EFA

deficiency would further increase bile flow, biliary bile salt output and pool size in *Fxr*$^{-/-}$ mice, compared with EFA deficient control mice. However, bile flow, biliary bile salt output and the bile salt pool size were similar in EFA deficient *Fxr*$^{-/-}$ and control mice. This observation suggests that either FXR inactivation or EFA deficiency maximally induces biliary bile salt output and pool size.

The fat absorption coefficient was higher in EFA deficient *Fxr*$^{-/-}$ mice compared to control mice, associated with a higher body weight gain in the former. Previous studies in EFA deficient wild type mice indicated that the fat malabsorption involves the mucosal phase of fat absorption, i.e, fatty acid translocation, triglyceride esterification and/or chylomicron formation [8]. We did not find indications for different mRNA expression levels of Fat, Ifabp-1, and Fatp-4, or of Dgat-1, Dgat-2 and Mttp in EFA deficient *Fxr*$^{-/-}$ and control mice. These results do not support the theoretical possibility that fatty acid transport across intestinal membranes, triglyceride re-esterification or chylomicron assembly account for the relatively preserved fat absorption in EFA deficient *Fxr*$^{-/-}$ mice. Our data do show, however, that inactivation of FXR was associated with an increase in cholic acid-muricholic acids ratio during EFA deficiency in mice. The increase in the cholic acid-muricholic acids ratio renders the bile salt pool more hydrophobic. The cholic acid-muricholic acids ratio (hydrophobicity of the bile salt pool) in bile has been positively associated with the capacity to absorb cholesterol in rats [24,25]. In analogy to these data on cholesterol absorption, we speculate that the higher cholic acid-muricholic acids ratio in EFA deficient *Fxr*$^{-/-}$ mice enhances the absorption of fatty acids and monoglycerides, possibly by facilitating their transfer across the unstirred water layer and thus their translocation across the apical membrane of the enterocytes. A higher cholic acid-muricholic acids ratio is found in EFA deficiency in mice (+63%), as well as in *Fxr*$^{-/-}$ mice (+77%), compared to the control situations. The increase in the cholic acid-muricholic acids ratio was not higher in EFA deficient *Fxr*$^{-/-}$ mice (+53%) compared to the increase in EFA deficient mice (+63%) or *Fxr*$^{-/-}$ mice (+77%), suggesting that FXR inactivation or EFA deficiency maximally increase the hydrophobicity of the bile salt pool [8,10]. In EFA deficient mice fat absorption was lower than in non EFA deficient mice, despite the high cholic acid-muricholic acids ratio in the former [8]. These observation suggests 1. that fat absorption would have been even more affected if the bile salt pool composition was not changed upon EFA deficiency; 2. that other factors than bile salt pool composition seem to contribute to EFA deficient fat malabsorption.

The mechanism underlying EFA deficiency-induced or FXR deficiency-induced increased hydrophobicity of the bile salt pool is still unclear. As discussed previously by Kok *et al*., it is counterintuitive that Cyp7a1 expression is increased and Cyp8b1 expression remains similar in *Fxr*$^{-/-}$ mice. Cyp8b1 catalyzes the 12α-hydroxylation of intermediates of the classic pathway of bile salt biosynthesis, leading to cholic acid biosynthesis and therefore appears to be important in determining the ratio between the ratio of cholic acid to chenodeoxycholic acid metabolites, the muricholic acids [26,27].

In conclusion, EFA deficiency in *Fxr*$^{-/-}$ mice causes milder fat malabsorption and a higher weight gain compared to *Fxr*$^{+/+}$ mice, probably related to the production of bile with more hydrophobic bile salts. These results support the theoretical concept that antagonists of FXR and/or increasing the hydrophobicity of the bile salt pool could ameliorate the phenotype of EFA deficiency in patients.

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CHAPTER 7

General discussion

GENERAL DISCUSSION

Cholestatic liver disease covers a wide range of conditions characterized by defective bile formation associated with reduced bile salt transport from the liver into the intestinal lumen. Physiological consequences include retention of bile salts and other bile consituents in the hepatocytes, limited availability of bile salts in the intestinal lumen and elevated plasma bile salt levels. These consequences eventually lead to liver injury, lipid malabsorption, pruritus, jaundice and potentially peripheral tissue injury. Cholestatic patients, especially children, frequently develop failure to thrive which consists of failure to grow and nutritional deficiencies, including those of fat-soluble vitamins and essential fatty acids (EFA). A malnutritional state strongly affects prognosis of cholestatic patients. Identification of nutritional deficiencies in cholestasis and understanding of the pathophysiology underlying these deficiencies will help to improve the prognosis of cholestatic children by allowing optimalization of their nutritional status. We therefore aimed to elucidate the effects of cholestasis and EFA deficiency on intestinal function, with emphasis on digestion and absorption of fats and carbohydrates.

Fat digestion and absorption in cholestatic conditions

Fat absorption is significantly impaired during cholestasis and EFA deficiency. In cholestatic disorders, EFA deficiency is induced by fat malabsorption. However, EFA deficiency itself is also able to induce fat malabsorption. In this way, cholestatic children can enter a 'vicious circle' of fat malabsorption inducing EFA deficiency and vice versa. Hence, unraveling the mechanisms behind cholestasis-induced fat malabsorption and EFA deficiency-induced fat malabsorption are of great importance.

Fat malabsorption in cholestasis is obviously to a great part due to limited availability of bile salts in the intestinal lumen, resulting in impaired fat solubilization. In chapter 3 we set out to address intestinal function in cholestasis. Our data indicate that fat absorption is impaired in cholestatic as well as in bile-deficient rats. Fat malabsorption in cholestatic and bile-deficient animals is at least for a large part caused by insufficient micellar solubilization due to limited availability of bile salts and phospholipids in the intestinal lumen. Interestingly, bile-deficient rats that do not show systemic accumulation of bile salts as seen in cholestasis, but also lack luminal bile salts, had milder fat malabsorption than cholestatic rats. Bile-deficient rats appear to compensate by ingesting more fat. Cholestatic and bile-deficient rats share the intestinal phenotype, but differ in the accumulation of bile salts in hepatocytes, cholangiocytes and circulation. We speculate that in absence of accumulation of bile salts, compensation mechanisms exist to counteract fat malabsorption.

The mechanism behind EFA deficiency induced fat malabsorption is less clear. EFA deficiency in rats has been ascribed to decreased bile flow and biliary secretion of bile salts and phospholipids, leading to impaired fat solubilization [1,2]. In addition, intracellular events such as triglyceride re-esterification and chylomicron formation were found to be impaired [1,3]. Fat digestion and fatty acid uptake by the enterocytes, however, were not affected [1,3]. In mice, data in literature about EFA deficiency-induced fat malabsorption only relates to bile formation. In contrast to rats, EFA deficiency in mice is not due to impaired bile formation. Rather, EFA deficiency in mice increases bile flow and biliary secretion of bile salts and phospholipids [4]. Cholestasis is associated with accumulation of bile salts in hepatocytes and cholangiocytes. Bile salts are natural ligands for the nuclear receptor FXR, which is involved in bile salt homeostasis and lipid homeostasis. Since bile salt homeostasis and lipid homeostasis are also altered during EFA deficiency [4], we wondered whether FXR is

involved in EFA deficiency-induced fat malabsorption. The FXR deficient and EFA deficient conditions separately have been associated with increased bile flow and bile salt pool size [4,5]. EFA deficient *Fxr*$^{-/-}$ mice had similar bile flow and similar bile salt pool sizes compared to EFA deficient control mice, suggesting that the bile flow and biliary bile salt output is maximally induced during EFA deficiency or FXR inactivation. Concluding, the amelioration of fat malabsorption in EFA deficient *Fxr*$^{-/-}$ mice compared to EFA deficient control mice is not due to altered bile flow.

In chapter 6, we suggested that altered composition of the bile salt pool might underly the improvement of fat malabsorption in EFA deficient *Fxr*$^{-/-}$ mice. The cholic acid to muricholic acids ratio was increased in EFA deficient *Fxr*$^{-/-}$ mice compared to EFA deficient mice, rendering the bile salt pool more hydrophobic. In rodents, the majority of the bile salt are conjugated with taurine. The order of taurine-conjugated bile salts ranging from relatively hydrophilic to relatively hydrophobic is: α-muricholic acid - β-muricholic acid – ursodeoxycholic acid – cholic acid – chenodeoxycholic acid. Increased hydrophobicity of the bile salt pool has been associated with increased absorption of cholesterol [6-9], probably due to enhanced solubilization of cholesterol and/or facilitated dissociation at the unstirred water layer. Increased solubilization and/or dissociation could also account for increased uptake of fatty acids, leading to amelioration of fat malabsorption as seen in EFA deficient *Fxr*$^{-/-}$ mice compared to EFA deficient control mice. This phenomenon was also observed in the separate deficiencies, EFA deficiency and FXR deficiency [4,5]. We can speculate about the mechanism underlying the increased hydrophobicity of the bile salt pool in absence of FXR and EFAs. The key enzyme regulating the hydrophobicity of the bile salt pool is sterol 12α-hydroxylase CYP8B1. CYP8B1 is required for the conversion of cholesterol to the primary bile salt cholic acid. The rate-controlling enzyme involved in bile salt biosynthesis CYP7A1 is required for synthesis of both cholic acid and chenodeoxycholic acid. In rodents, the majority of chenodeoxycholic acid is converted to α-muricholic acid, which is subsequently converted to β-muricholic acid. Thus the CYP8B1 activity level relative to the CYP7A1 activity level determines the hydrophobicity of the bile salt pool. Kok *et al.* demonstrated that FXR inactivation in mice was associated with increased mRNA expression of Cyp7a1, while mRNA expression of Cyp8b1 was similar in *Fxr*$^{-/-}$ mice compared and control mice. The ratio of Cyp7a1 to Cyp8b1 expression, however, was increased, suggesting a shift of the balance towards chenodeoxycholic acid and muricholic acids synthesis rather than cholic acid [5]. We speculate that Cyp8b1 may not be involved in regulation of bile salt pool hydrophobicity.

In cholestatic animals, in contrast, the cholic acid to muricholic acid ratio is decreased, leading to increased hydrophilicity of the bile salt pool (unpublished observations). Concomitantly, CYP8B1 expression has been shown to be repressed in cholestatic rats [10]. Thus, the hydrophobicity of the bile salt pool is decreased in cholestatic animals to counteract hydrophobic bile salt-induced cytotoxicity and the hydrophobicity of the bile salt pool is increased in EFA deficient animals to counteract fat malabsorption.

Interestingly, FXR inactivation and inactivation of its target gene SHP have been associated with protection against bile salt-induced liver injury [11,12]. Thus, removal of FXR or its ligands, bile salts, results in an improvement in fat absorption and protection against bile salt-induced liver injury. Since bile salts and FXR are involved in many other physiological functions, such as energy homeostasis and glucose homeostasis [13], antagonism of FXR would likely lead to many side effects. However, if we can unravel which mechanisms underly the amelioration of fat malabsorption and hepatoprotection in EFA deficient *Fxr*$^{-/-}$ mice compared to EFA

deficient control mice and bile-deficient rats compared to cholestatic rats, we might have a target to intervene clinically.

Carbohydrate digestion and absorption in cholestatic conditions

Defects in bile production, as observed in cholestasis, limit the availability of bile salts in the intestinal lumen for the facililation of lipid-soluble nutrient absorption. Another aspect of cholestasis is a strongly increased plasma bile salt level, due to regurgitation of bile salts into the circulation from the hepatocytes [14-16]. We wondered whether this high plasma bile salt level could affect intestinal epithelial cells through processes such as proliferation, differentiation or apoptosis, eventually leading to altered intestinal function. There have been numerous reports demonstrating that bile salts can induce proliferation, differentiation or apoptosis *in vitro*, depending on their hydrophobicity, conjugative state, concentration, cell signaling pathways and cell type involved [17-32].

As cholestatic liver disease is frequently accompanied by nutritional defects [16], we aimed to explore intestinal digestion and absorption of carbohydrates in cholestatic conditions. *In vivo* data about intestinal function during cholestasis, specifically carbohydrate digestion or absorption is scarce. Jejunal absorption of glucose was found unaffected in cholestatic rats [33]. Also bile-deficiency alone, i.e. without cholestasis did not impair sucrase enzyme activity in rats [34], suggesting that intestinal nutrient absorption is maintained in animal models of intestinal bile deficiency. We addressed whether carbohydrate digestion was affected in cholestatic rats (chapter 3). Interestingly, carbohydrate digestion was maintained in cholestatic rats, but impaired in EFA-deficient mice (chapter 5). This discrepancy can be explained by the fact that the cholestatic rats were subjected to the carbohydrate digestion and absorption test 1 week after bile duct ligation, which is too short to develop EFA deficiency. Concluding from chapter 3, 4 and 5 it is highly likely that lactose digestion is impaired in cholestatic disorders that are accompanied by EFA deficiency.

Enterocytes are protected during cholestasis, both in *in vitro* (chapter 4) and *in vivo* (chapter 3). In chapter 4 we propose that nutrient absorption is not affected in cholestatic conditions, because nutrient absorption and bile salt re-absorption are localized in different intestinal segments. The jejunum is the major site of nutrient absorption, while bile salt re-absorption is restricted to the terminal ileum [35]. We demonstrated that reduced sucrase activity was associated with increased expression of intestinal bile salt transporters (ASBT). Concluding, intestinal function, i.e. sucrase activity, is impaired when high amounts of bile salt are transported into the enterocytes. In accordance with this theory, Lee *et al.* showed that mRNA and activity levels of sucrase were strongly reduced in the terminal ileum compared to the duodenum, jejunum and proximal ileum [36]. That the separation of nutrient absorption and bile salt re-absorption in the intestine is highly important and tightly regulated was also demonstrated by Bosse *et al.* The authors identified the transcription factor Gata-4 as the major determinant of jejunal-ileal identities in mice. Synthesis of a transcriptionally inactive Gata-4 mutant in the mouse jejunum resulted in an attenuation of expression of genes involved in nutrient absorption and an induction of genes involved in bile salt re-absorption [35].

Snipes *et al.* noted pathological changes in the intestines of EFA deficient rats, i.e. restricted surface area due to villi shortening and a lack of cellular differentiation [37]. This effect has been ascribed to the decreased EFA content in membrane phospholipids [1,3]. Our study with EFA deficient mice also pinpointed the defect in lactose digestion to the jejunum, though not accompanied by villi shortening (chapter 5). Interestingly, a significant difference in the EFA

linoleic acid (LA) concentration was observed between jejunal membrane phospholipids of EFA deficient and control mice. Cell membrane fluidity is determined by its lipid composition. Incorporation of saturated fatty acids and cholesterol in the membrane will render the membrane more rigid, while incorporation of unsaturated fatty acids will make it more fluid. The number of receptors and their affinity to their respective ligands is considered to depend on the fluidity of the cell membrane [38]. A reduction in plasma membrane fluidity has also been associated with decreased luminal membrane permeability to macromolecules [39]. Moreover, decreased incorporation of EFA-derived fatty acids in membrane phospholipids has been associated with altered activity of hydrolytic enzymes in the jejunum [40]. This is in accordance with the observation that lactose digestion was impaired in EFA deficient mice. In contrast to lactose digestion, glucose absorption was maintained in EFA deficient mice. The altered composition of membrane fatty acids may only affect activity of hydrolytic enzymes such as lactase that are attached to the membrane [41], while the membrane-spanning glucose transporters are unaffected. However, it should be realized that not only the activity of lactase was decreased in EFA deficient jejunum, but also its mRNA expression. This observation indicates that the effects of EFA deficiency cannot completely be explained by alterations of the cell membrane fluidity.

Concluding remarks and perspectives

In conclusion, cholestasis as such does not lead to impaired intestinal carbohydrate digestion. When accompanied by EFA deficiency, however, cholestasis in rats is also associated with impaired lactose digestion. Thus, our initial conclusion (chapter 3) that carbohydrate addition to the diet of cholestatic patients should be valuable for optimalization of their nutritional status has to be fine-tuned. Addition of monosaccharides to the diet of cholestatic patients is not be beneficial, since they have a high glycemic index. However, by adding small amounts of disaccharides more frequently per day, we may be able to maximally utilize the limited carbohydrate digestion capacity.

In order to assess carbohydrate digestion and absorption in an animal model we adapted an existing test based on stable isotope dilution technique [42,43]. The blood spot technique, developed by our group, made it possible to test these parameters in mice [44]. Combination of both techniques makes it possible to test digestion and absorption of various nutritional compounds using small volumes of blood, and can thus be utilized for small animals and neonates. The development and adaptation of this technique to specific nutrients, organisms and disorders, will accelerate the identification of specific nutritional deficiencies in cholestatic children

Regarding the role of FXR in therapies for EFA deficiency, FXR has been implicated in so many regulatory functions that antagonizing FXR solely for the purpose of enhancing fat absorption is not applicable. Perhaps, if we identify the mechanism behind the FXR inactivation-induced amelioration of fat malabsorption, we can aim to develop gene-selective or organ-selective ligands to specifically target the gene or organ responsible.

In this thesis we gained more insight in the pathophysiology of cholestasis-induced failure to thrive. On this account, we can aim to further optimize the nutritional status of children with cholestatic liver disease.

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SUMMARY

Cholestatic liver disease covers a wide range of conditions characterized by defective bile formation associated with reduced bile salt transport from the liver into the intestinal lumen. Physiological consequences include retention of bile salts and other bile consituents in the hepatocytes, limited availability of bile salts in the intestinal lumen and elevated plasma bile salt levels. These consequences eventually lead to liver injury, lipid malabsorption, pruritus, jaundice and potentially peripheral tissue injury. Cholestatic patients, especially children, frequently develop failure to thrive which consists of failure to grow and nutritional deficiencies, including those of fat-soluble vitamins and essential fatty acids (EFA). A malnutritional state strongly affects prognosis of cholestatic patients. Identification of nutritional deficiencies in cholestasis and understanding of the pathophysiology underlying these deficiencies will help to improve the prognosis of cholestatic children by allowing optimalization of their nutritional status. We therefore aimed to elucidate the effects of cholestasis and EFA deficiency on intestinal function, with emphasis on digestion and absorption of fats and carbohydrates.

Chapter 2 relates to the nutritional deficiencies that accompany cholestatic liver disease in children as well as treatment options based on nutrient supplementation. Important factors in the pathophysiology of malnutrition in pediatric cholestasis are poor dietary intake, increased energy expenditure and impaired absorption of fats and fat-soluble nutrients. To counteract malnutrition in cholestasis, dietary energy intake in cholestatic children is usually increased to 120-150% of the recommended daily energy intake. In addition, up to 60% of the fat components, particularly long-chain triglycerides are substituted by medium-chain triglycerides whose absorption occurs relatively independent from the presence of bile components in the intestinal lumen. Pediatric cholestatic patients comprise a heterogeneous group: consequently clinical manifestations of the disease may vary widely. This makes a tailor-made dietary approach for these children crucial.

As stated above, cholestasis is often accompanied by a strongly elevated plasma bile salt level. Many reports have demonstrated that bile salts can induce cellular proliferation or apoptosis, depending on the bile salt species, concentration and cell type involved. We have evaluated whether elevated plasma bile salt concentrations could affect intestinal function, for instance by affecting proliferation, differentiation or apoptosis. In **chapter 3**, we investigated the effect of cholestasis on intestinal function, i.e., on digestion and absorption of sucrose and glucose in rats, with stable isotope methodology. We compared cholestatic rats to control rats and bile-deficient rats, to be able to differentiate between consequences of elevated plasma bile salts and absence of bile from the intestine. Intestinal sucrose digestion and glucose absorption occurred to a similar extent in cholestatic, control and bile-deficient, indicating that this aspect of intestinal function, i.e., carbohydrate digestion and absorption, is preserved in cholestatic conditions.

In **chapter 4**, we studied the effect of conjugated bile salts in cholestatic concentrations on intestinal epithelial cells *in vitro*. The human colon carcinoma cell line Caco-2, which develops small intestinal characteristics upon differentiation, was exposed to cholestatic conditions at different developmental stages. Exposure of proliferating or short-term differentiative cells to conjugated bile salts in cholestatic concentrations did not affect

intestinal cell proliferation, differentiation or apoptosis, indicating that intestinal cells in the respective developmental stages are resistant to cholestatic conditions. Exposure of long-term differentiative cells to cholestatic conditions, however, resulted in decreased sucrase activity, coinciding with increased expression of the bile salt transporter ASBT. Nutrient absorption, including sucrase activity, is most efficient in the jejunum, while active bile salt absorption is restricted to the terminal ileum. We speculated that enterocytes are protected from bile salt-induced effect through absence of the intestinal bile salt transporter ASBT.

Cholestasis-induced fat malabsorption often leads to EFA deficiency. EFA deficiency in itself, however, can also induce fat malabsorption. The pathophysiological basis of EFA deficiency-induced fat malabsorption is incompletely understood, but is probably located at the mucosal level. We have evaluated whether EFA deficiency would also affect carbohydrate digestion or absorption. In **chapter 5**, we tested this theory by subjecting EFA deficient mice to a lactose digestion/glucose absorption test using stable isotope methodology. Lactose digestion, but not glucose absorption, was impaired in EFA deficient mice. Impaired lactose digestion coincided with decreased activity and mRNA levels of lactase in the jejunum. In addition, we found a positive correlation between jejunal lactase activity and membrane phospholipid linoleic acid content, suggesting that altered fatty acid composition of cellular membranes affects lactose digestion during EFA deficiency.

The farnesoid X receptor (FXR) has been implicated in the regulation of bile salt and lipid metabolism. Fxr-deficient mice have increased bile flow and bile salt pool size, and a more hydrophobic bile salt composition due to an increased contribution of cholic acid (CA). Interestingly, we previously demonstrated that EFA-deficiency in (wild-type) mice has similar phenotypic characteristics. Based on this similarity, we wondered whether FXR could be involved in EFA deficiency-induced fat malabsorption. In **chapter 6**, we assessed fat absorption and bile salt homeostasis parameters in EFA deficient *Fxr*$^{-/-}$ mice compared to EFA deficient control mice. Inactivation of FXR resulted in milder fat malabsorption and increased weight gain, coinciding with increased hydrophobicity of the bile salt pool. Thus, FXR appears to be involved in EFA deficiency-induced fat malabsorption, possibly by modulating the hydrophobicity of the bile salt pool.

According to the work described in this thesis, cholestasis when accompanied by EFA deficiency seems to have a clear negative influence on intestinal function. This information allows us to develop strategies to optimize the nutritional status of cholestatic patients and thereby improve their prognosis.

NEDERLANDSE SAMENVATTING

Cholestatische leverziekten worden gekarakteriseerd door verminderd transport van gal van de lever naar het darmlumen. Fysiologische gevolgen hiervan zijn de accumulatie van galzouten en andere galcomponenten in bloed, lever en andere organen, en verminderde beschikbaarheid van galzouten in het darmlumen. Cholestatische leverziekten zijn dan ook vaak geassocieerd met leverschade, vetmalabsorptie, jeuk en geelzucht. Kinderen met een cholestatische leverziekte ontwikkelen vaak het zogenaamde “failure to thrive”; gestoorde groei en deficiënties van belangrijke voedingsstoffen, inclusief vet-oplosbare vitamines en essentiële vetzuren (EFA). Een slechte voedingsstatus heeft een sterk effect op de prognose van cholestatische patiënten. Identificatie van de specifieke deficiënties die optreden tijdens cholestase en het ophelderen van de onderliggende pathofysiologie zal bijdragen aan het optimaliseren van de voedingsstatus van cholestatische kinderen. In dit proefschrift is onderzoek beschreven met betrekking tot de effecten van cholestase en EFA deficiëntie op de darmfunctie, met de nadruk op de digestie en absorptie van vetten en koolhydraten.

In **hoofdstuk 2** worden de nutritionele deficiënties besproken die optreden bij cholestatische leverziekten in kinderen, evenals behandelingstrategieeën gebaseerd op voedingssupplementen. De ondervoeding die optreedt tijdens cholestase wordt vaak veroorzaakt door anorexia, verhoogd energieverbruik en sterk verminderde absorptie van vetten en vet-oplosbare nutriënten. Om dit te ondervangen wordt de energiewaarde van het voedsel dat cholestatische kinderen moeten innemen, verhoogt tot 120-150% van de geadviseerde dagelijkse hoeveelheid. Tot 60% van de vetcomponenten, de lange keten vetzuren, worden vervangen door medium keten vetzuren, die onafhankelijk van de aanwezigheid van galcomponenten in het darmlumen worden opgenomen. Pediatrische cholestatische patiënten vormen een heterogene groep, waarvan de klinische manifestaties van de aandoening sterk variëren. Dit maakt een toegepaste aanpak voor deze kinderen cruciaal.

Cholestase wordt vaak vergezeld door een sterk verhoogd galzoutconcentratie in het plasma. Galzouten kunnen cellulaire proliferatie, differentiatie en apoptosis induceren, afhankelijk van het specifieke galzout, concentratie en het betrokken celtype. We hebben onderzocht of de verhoogde galzoutconcentratie in het plasma de darmfunctie beïnvloedt, bijvoorbeeld door proliferatie, differentiatie of apoptosis te beïnvloeden. In **hoofdstuk 3** hebben we het effect van cholestase op de darmfunctie onderzocht, specifiek op de splitsingscapaciteit en absorptie van sucrose en glucose in ratten met behulp van stabiele isotopen methodologie. We hebben cholestatische ratten vergeleken met galdeficiënte ratten, om te kunnen discrimineren tussen de gevolgen van de verhoogde plasma galzoutconcentratie en de afwezigheid van galzouten in de darm. Sucrose digestie en glucose absorptie in de darm verliep in gelijke mate in cholestatische, controle en galdeficiënte ratten. Dit wijst erop dat koolhydraat splitsing en absorptie geconserveerd zijn tijdens cholestase.

In **hoofdstuk 4** hebben we het effect van geconjugeerde galzouten in cholestatische concentraties op epithele darmcellen *in vitro* onderzocht. De humane colon carcinoma cellijn Caco-2, welke dunne darm karakteristieken ontwikkelt wanneer de cellen differentiëren,

werd blootgesteld aan cholestatische condities in verschillende ontwikkelingsfases. Blootstelling van prolifererende cellen en korte-termijn gedifferentieerde cellen aan geconjugeerde galzouten in cholestatische concentraties had geen effect op Caco-2 proliferatie, differentiatie of apoptose. Dit duidt op resistentie van de darmcellen tegen blootstelling aan galzouten in cholestatische concentraties. Blootstelling van lange-termijn gedifferentieerde cellen daarentegen, resulteerde in een reductie van de splitsingscapaciteit van het brush-border enzym sucrase gelijktijdig met verhoogde expressie van de galzouttransporter ASBT. Opname van nutriënten, inclusief de splitsingscapaciteit van sucrase, is het meest efficiënt in het jejunum, terwijl actieve absorptie van galzouten plaatsvindt in het terminale ileum. We speculeren dat enterocyten die betrokken zijn bij de absorptie van nutriënten worden beschermd tegen cholestatische condities door de afwezigheid van de galzouttransporter ASBT.

Vetmalabsorptie, veroorzaakt door cholestase, leidt vaak tot EFA deficiëntie. EFA deficiëntie kan echter zelf ook vetmalabsorptie induceren. De pathofysiologische basis van de door EFA deficiëntie geïnduceerde vetmalabsorptie is nog niet opgehelderd, maar is waarschijnlijk gelokaliseerd op het mucosale niveau. We hebben onderzocht of EFA deficiëntie een effect heeft op koolhydraat splitsing of absorptie. In **hoofdstuk 5** hebben we dit onderzocht door EFA deficiënte muizen te onderwerpen aan een gecombineerde lactose splitsing/glucose absorptie test, gebruik makend van stabiele isotopen methodologie. In tegenstelling tot glucose absorptie, was de lactose splitsing sterk verminderd in EFA deficiënte muizen tegelijk met verlaagde activiteit en mRNA niveaus van lactase in het jejunum. Tevens vonden we een positieve correlatie tussen lactase activiteit in het jenunum en het aandeel van linolzuur in membraan phospholipiden. Dit wijst erop dat een veranderde compositie van vetzuren in het cellulaire membrane de lactose splitsing tijdens EFA deficiëntie beïnvloedt.

De farnesoid X receptor (FXR) is betrokken bij de regulatie van galzout- en vetmetabolisme. Fxr-deficiënte muizen zijn gekarakteriseerd door een verhoogde galflow en galzoutpool, evenals een verhoogd aandeel van cholaat in de galzoutpool en daardoor meer hydrofobe compositie. Voorheen hebben we aangetoond dat EFA deficiëntie in wild-type muizen vergelijkbare karakteristieken heeft. Gebaseerd op deze gelijkenis, vroegen wij ons af of FXR betrokken is bij vetmalabsorptie geïnduceerd door EFA deficiëntie. In **hoofdstuk 6** hebben we dan ook gekeken naar vetabsorptie en galzouthomeostase parameters in EFA deficiënte *Fxr*$^{-/-}$ muizen vergeleken met EFA deficiënte controle muizen. Inactivatie van FXR resulteerde in een mildere vetmalabsorptie en verhoogde gewichtstoename, tegelijk met verhoogde hydrofobiciteit van de galzoutpool. Concluderend, FXR lijkt betrokken bij vet malabsorptie geïnduceerd door EFA deficiëntie, waarschijnlijk door middel van modulatie van de hydrophobiciteit van de galzoutpool.

Op grond van deze bevindingen lijkt cholestase in combinatie met EFA deficiëntie een duidelijk negatieve invloed uit te oefenen op het functioneren van de darm. Deze informatie stelt in staat om rationele voedingsaanpassingen te maken om de voedingsstatus en daarmee de prognose van cholestatische patiënten te verbeteren.

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Liefs Leo(nie)

CURRICULUM VITAE

Leonie Los werd op 26 februari 1980 geboren in Zwolle. Na het behalen van haar VWO diploma aan het Carolus Clusius College te Zwolle begon zij in 1998 met de studie (Medische) Biologie aan de Rijksuniversiteit Groningen. Het eerste wetenschappelijk onderzoek werd uitgevoerd binnen de vakgroep Hematologie van Prof. dr. E. Vellenga en betrof onderzoek naar STAT5 en MAPK/ERK signaaltransductie in TPO-gestimuleerde megakaryocyten en EPO-gestimuleerde erythrocyten. Het tweede onderzoek werd uitgevoerd binnen de vakgroep Immunologie in Verona (Italië) onder begeleiding van Prof. dr. M. Colombatti. Het onderzoek betrof de optimalizatie van de koppeling van de toxine saporin aan antilichamen specifiek voor kankercellen. Eind augustus 2003 studeerde Leonie af en begon met promotie-onderzoek bij de vakgroep Kindergeneeskunde van de medische faculteit van de Rijksuniversiteit Groningen onder begeleiding van Prof. dr. H.J. Verkade, Prof. dr. F. Kuipers en Dr. E.H.H.M. Rings. De resultaten van het onderzoek uitgevoerd gedurende deze periode staan beschreven in dit proefschrift.

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A.L. Drayer, A.K. Boer, **E.L. Los**, M.T. Esselink, E. Vellenga. Stem cell factor synergistically enhances thrombopoietin-induced STAT5 signaling in megakaryocyte progenitors through JAK2 and Src kinase. *Stem Cells.* 2005: 23: 240-251.

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